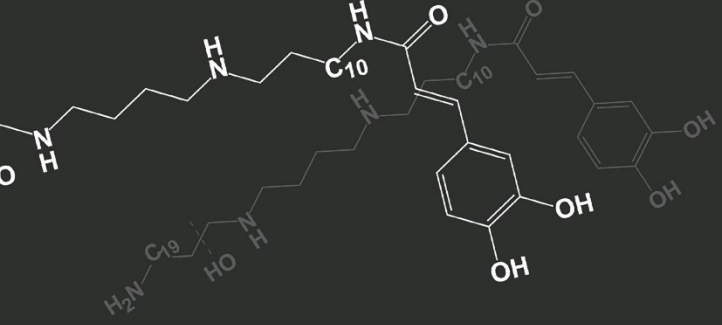
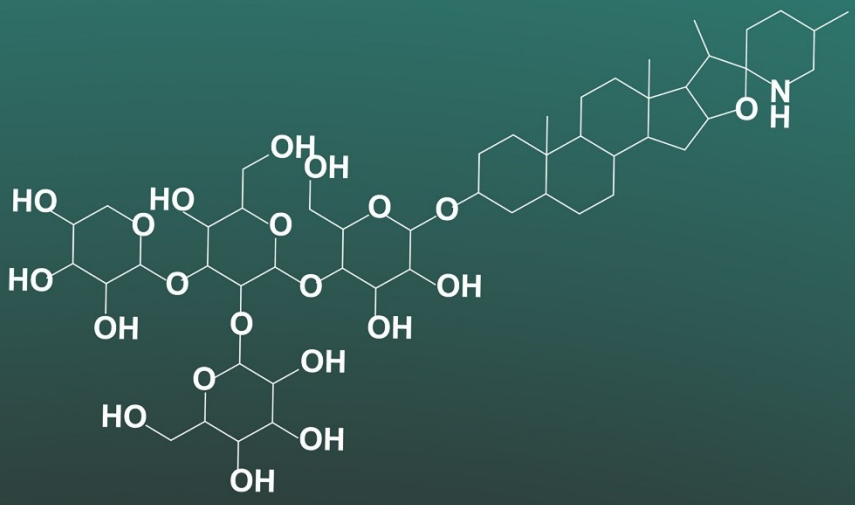
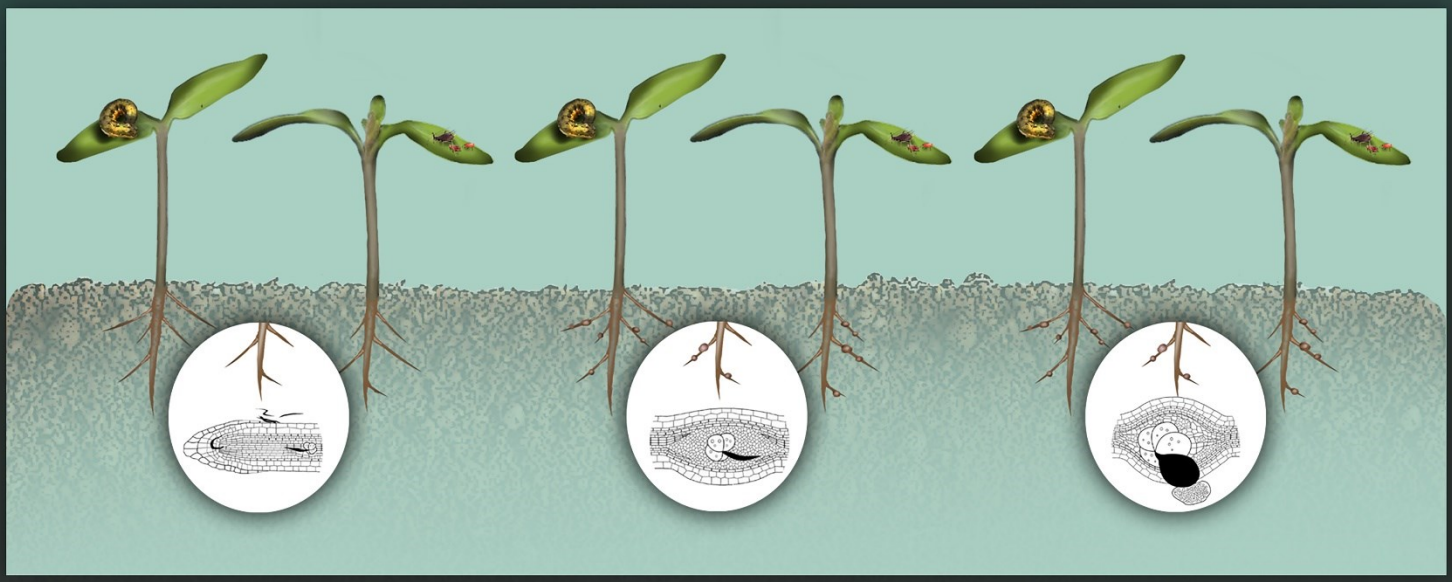


Crispus M. Mbaluto

# Deciphering systemically induced plant responses underlying interactions between root-knot nematode and shoot herbivores





**Printed and/or published with the support of the German Academic Exchange Service**

# Deciphering systemically induced plant responses underlying interactions between root-knot nematode and shoot herbivores

DISSERTATION

zur Erlangung des akademischen Grades
**„doctor rerum naturalium“ (Dr. rer. nat.)**

**vorgelegt dem Rat der Fakultät für Biowissenschaften**
**der Friedrich-Schiller- Universität Jena**

von
**Crispus Musyoki Mbaluto, M.Sc. Biotechnology**
geboren am 10.01.1984 in Machakos, Kenia

**Gutachter:**

**Prof. Dr. Nicole M. van Dam:**

Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, and Institute of Biodiversity, Faculty of Biological Sciences, Friedrich-Schiller-Universität Jena, Germany

**Prof. Dr. Carolina Escobar:**

Department of Environmental Sciences, Faculty of Environmental Sciences and Biochemistry, University of Castilla La Mancha, Toledo, Spain

**Dr. Ainhoa Martinez-Medina:**

Plant-Microorganism Interaction, Institute of Natural Resources and Agrobiology of Salamanca (IRNASA-CSIC), Salamanca, Spain

*Beginn der Promotion:* ***04.10.2016***

*Eingereicht am:* ***26.06.2020***

*Tag der öffentlichen Verteidigung:* ***21.10.2020***

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# CHAPTER 1

# General introduction

## 1.1 Ecological and agro-economic importance of insect pests

Plants are the primary foundation of virtually all terrestrial food networks. Each of the more than 300,000 plant species interacts with a range of organisms belonging to different kingdoms, including animals, microbes (i.e., beneficial, symbiotic, pathogenic), and arthropods (van der Ent et al., 2009). Insects form the largest and most diverse group of organisms living on earth and occur in all ecosystems, except in open oceans (May, 1992; Douglas, 2018; Stork, 2018). Plants and insects have co-existed for over 400 million years (Labandeira, 2013). Their interaction occurs belowground (BG) and aboveground (AG), and can be beneficial to both plants and insects. For example, during pollination, insects move within and among flowers to obtain nectar and, at the same time, transfer pollen (Calderone, 2012; Rader et al., 2016; Hung et al., 2018). Moreover, insects disperse seeds when flying from one location to another (Pellmry, 1985; Chen et al., 2017). Another beneficial relationship involves the recruitment of natural enemies to herbivorous insects that damage plants. Indeed, plants under attack can recruit natural enemies to the specific attacker. The natural enemies catch and eat their prey or gain access to a host for oviposition, while the plant benefits from reduced herbivory (Turlings et al., 1990, 2012; Poelman et al., 2012; Laznik and Trdan, 2016; Mechaber, 2020). Besides the beneficial interactions, detrimental interactions between plants and insect pests are frequent as well. For instance, from the plant's perspective, insect herbivory and transmission of diseases reduce the plant's performance (Chowański et al., 2016; Mechaber, 2020). Detrimental BG and AG insect pests can devastate life-sustaining crops and plants, and tremendously affect the economics of agricultural practices. Overall, it is estimated that insect pests cause approximately 45 % losses in annual food production worthy hundreds of billions of dollars (Sharma et al., 2019). Conventional crop protection strategies such as resistance breeding and the use of pesticides are limited in terms of sustainability. This is because insect pests can develop resistance against these control methods. Moreover, several pesticides are currently banned from use due to multiple negative effects on human health and the environment (Franco et al., 2015; Borel, 2017). The endeavor of agriculture requires sustainable crop protection methods for predictable and economical food production. To this end, efforts to identify natural plant resistance traits for AG and BG insect pests may help to develop sustainable pest management strategies, e.g., breeding crops with improved resistance against insect pests, as portrayed (Figure 1).

## 1.2 Plant defense against insect pests and pathogens

Plants exist in a dynamic environment and encounter constant pressures from biotic stressors, including pathogens, parasitic nematodes, and insect herbivores, which are a threat to the plant's growth and development. To defend themselves, plants produce vast and complex arrays of defensive responses. There are many different types of defense responses, just as there are numerous different ways that plants can be attacked (Gurevitch et al., 2002; Mechaber, 2020). Plant defense strategies can be categorized into

three broad groups: deterrence, resistance, and tolerance. The deterrence strategies demotivate the insect pests from attacking a plant; -for example, they can originate from colors, odors, textures, or even absence of stimuli that otherwise would trigger herbivory or feeding. Resistance strategies include structural and or chemical traits that result in death, reduced performance and fecundity of the insect herbivores and maximize plant fitness. Tolerance is described as a strategy that does not primarily serve to interact with the insect herbivores negatively but help the plant to compensate for the damage via changes in assimilation rate, growth, resources allocation et cetera (Strauss and Agrawal, 1999; Tiffin, 2000; Schwachtje et al., 2006; Gómez et al., 2012; Robert et al., 2015). It is important to remark that these three strategies can overlap mechanistically and functionally, and therefore it is difficult to tell them apart. However, as a general rule, it is widely accepted that high insect pests pressure is mainly regulated via resistance, whereas low insect pests pressure is tolerated up to a certain threshold (Mitchell et al., 2016).

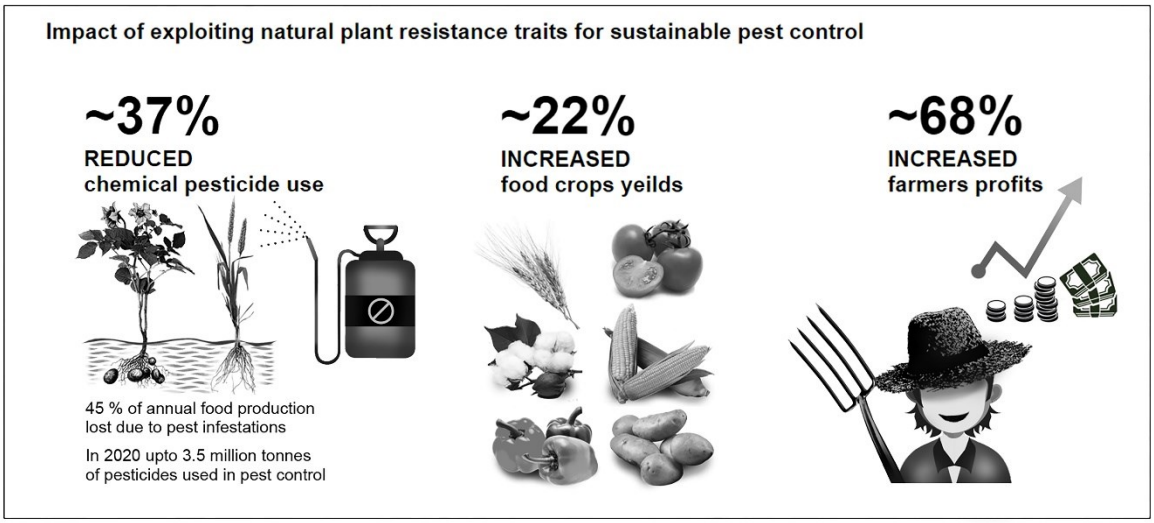


**Figure 1: Impact of exploiting natural plant defense mechanisms for sustainable pest control**. This illustration is based on the information and data published by Klümper and Qaim (2014) and Sharma et al. (2019). The exploitation of natural defense traits (biological control) that enable plants to resist insect pests attack can reduce the reliance on synthetic pesticides by approximately 37 %. Whereas reduced pesticide use alleviates adverse chemical effects on the environment and human health, farmers can also realize increased crop yields by approximately 22%. Trade on crop yields combined with the would-be expense for purchasing chemical pesticides can translate to about 68 % in profits for the farmers. Jennifer Gabriel did artwork of the figure.

Plant resistance traits or defense mechanisms are expressed constitutively and can be induced upon attack. Constitutive traits are physical and chemical barriers that are synthesized by plants all the time

(Wittstock and Gershenzon, 2002), whereas the inducible traits are responses triggered by the plants after an attack (Harvell, 1990). However, constitutive defenses can also increase when the plant is attacked. For example, in tomato, insect herbivory, and exogenous treatment with methyl jasmonate (MeJA) increase the number of trichomes compared to plants without herbivory or chemical treatment (Tian et al., 2012). A comparison between constitutive and inducible defense responses shows that inducible responses are less costly to the host plant and are more specific (Koricheva et al., 2004). It has been established that induction of defense response is a three-step process including (1) surveillance (detection of attack via specific recognition signals) (Mithöfer and Boland, 2008; Sanabria et al., 2010; Saijo et al., 2018), (2) signal transduction (transduction of detected signals via a network of signal transduction pathways) (Mulligan et al., 1997; Clark et al., 2001; Xing and Laroche, 2011), and (3) production of defense components, for example, secondary metabolites such as glucosinolates, alkaloids, *et cetera* (Fontaine and Irving Jr, 1948; Facchini, 2001; Hopkins et al., 2008; Singh, 2017). Inducible defenses are further grouped into two categories, direct and indirect defenses. Direct defenses are the phenotypic traits that by themselves reduce the susceptibility of the host plant to insect pests herbivory (Chen, 2008). Indirect defenses do not affect the susceptibility of the plant itself but promote the efficiency of natural enemies of the attacking insects (Heil and Ton, 2008; Aljbory and Chen, 2018). In this project, I mainly investigated the performance of insect herbivores as an effect of direct defenses. Therefore, I focus mainly on the inducible direct defenses in the remainder of this introduction.

Direct resistance traits are categorized into physical/mechanical features and chemical compounds. The physical traits include thorns, trichomes, cell wall fortification, and primarily function as pre-ingestion mechanisms that limit food supply to the attacking insect pests (reviewed by; Chen 2008; War *et al.* 2012; Mitchell *et al.* 2016). Chemical defenses consist of specialized bioactive compounds that cause physical damage and disrupt the physiology of the attacking insect pests (Chen, 2008; Howe and Schaller, 2008; Mitchell et al., 2016). From this point onwards, I will narrow the focus to discuss the chemical defenses.

In situations where the physical barriers fail to prevent feeding and damage by insect herbivores, the plants can resort to different sets of post-ingestion defense mechanisms that impair the performance of the attacking insect herbivores. One way is by interfering with primary metabolism. The primary metabolic pathway synthesizes vital compounds for the plant's growth, development, and production of cells (Pott et al., 2019). Some of the primary compounds, particularly carbohydrates and proteins (amino acids), are vital nutrients for insect growth and development (Behmer, 2009; Le Gall and Behmer, 2014; Wang et al., 2018b). Changes in the concentrations of these metabolites after the plant is attacked can influence the performance of the attacking insect. For example, the addition of high galactose concentration in the agar diet for western spruce budworm showed high larval mortality and less weight gain, while the presence of glucose and fructose enhanced the larvae growth and weight gain (Zou and Cates, 1994). Proteins (amino

acids) are the main form of nitrogen in plants and can limit the performance of insect herbivores. Nitrogen is a crucial nutrient for the majority of insects, and therefore insects can manipulate amino acids metabolism for their own benefit. For example, the wheat aphid *Schizaphis graminum* herbivory increases the levels of essential amino acids in the phloem sap of wheat (Dorschner et al., 1987; Sandström et al., 2000). Elevated levels of amino acids imply increased efficiency of nitrogen utilization and thus enhance the performance of aphids (Koyama et al., 2004; Faria et al., 2007) and caterpillars (Lee, 2007; Merkx-Jacques et al., 2008). Besides the changes in the levels of primary metabolites, herbivory can alter the primary metabolism when the plant's photosynthesis efficiency is enhanced or reduced. Moreover, herbivory can cause the remobilization of resources (carbon and nitrogen), and this can alter the plant growth rate and the performance of insect herbivores (Zhou et al., 2015). It can be interpreted that, for example, the mobilization of resources to damaged tissues is either to reinforce the induction of defenses locally or to manipulate the insect herbivore metabolism. On the other hand, the movement of resources away from the damaged tissues might imply that plants tolerate insect herbivores by preserving resources for later growth or by starving the herbivores (Zhou et al., 2015).

Next to changes in carbohydrates and proteins (amino acids), plants produce enzymes that stay stable and active inside the insect's gut after ingestion of plant tissues. These enzymes degrade the ingested essential nutrients that could otherwise benefit the insect in growth and performance. Some examples of these plants' enzymes include amino acid deaminases that degrade the ingested amino acids. For example, several studies show that threonine and arginine deaminases are activated in tomato upon herbivory by *Manduca sexta* and *Trichoplusia ni,* where they catabolize the essential amino acids; threonine and arginine in the insect's gut (Chen et al., 2005, 2007; Felton, 2005). Inhibition of the insect herbivore's digestive enzymes is another way to reduce nutritional value. Plants synthesize *proteinase inhibitors (PIs)* such as proteases and *α*-amylases that, upon insect attack, inhibit the insect's digestive enzymes (Chen, 2008). Early studies by Johnson and co-workers (1989) revealed that overexpression of *PIs* in transgenic tobacco plants reduced the development of *M. sexta* caterpillars (Johnson et al., 1989). Later studies confirmed the importance of *PIs* in different plant species against insect herbivores, mainly chewing herbivores (reviewed by; Erb and Reymond 2019). In the case of piercing-sucking herbivores, the role of defense proteins is not well studied. However, overexpression of the phloem protein gene *AtPP2-A1* in *Arabidopsis thaliana* causes clogging in sieve pores and represses the infestation of green peach aphid (Zhang et al., 2011; Kloth et al., 2017). Another recently studied protein is the SIEVE ELEMNT-LINING CHAPERONE1 which restricts the aphid stylet from piercing into the sieve elements in *A. thaliana* (Kloth et al., 2017).

Secondary metabolites are organic compounds that are important for plant's survival in the wild but are not critical for the plant's growth and development. Whereas the principal ecological function of secondary metabolites is the provision of chemical defense against insect pests and pathogens (Howe and

Schaller, 2008), they are also involved in other physiological functions including transport and storage of nitrogen, as well as the attraction of pollinators and seed dispersers (Wink, 2010; Luis et al., 2013). Secondary metabolites are diverse and phylogenetically distributed (Wink, 2003). Four principal groupings have been deduced based on their biosynthesis comprising terpenes, phenolics, sulfur-nitrogen metabolites, and nitrogen-based metabolites (Howe and Schaller, 2008). These groups have been reviewed extensively (Mazid et al., 2011; Zaynab et al., 2018), and therefore only I provide a brief highlight for each group. Terpenes constitute the largest group of secondary metabolites. They are commonly synthesized from isoprene units (Zhang et al., 2017). The majority of terpenes act as indirect plant defenses attracting natural enemies to insect herbivores (Kant et al., 2009). Phenolics are characterized by the presence of phenol, hydroxyl, and an aromatic phenyl ring (Quideau et al., 2011; Lattanzio, 2013). They are important in the defense against BG and AG feeding insect pests (Golan et al., 2017). Besides, they play other roles such as protecting the plant from UV radiation, pollen development, the attraction of pollinators, and seed dispersers (Kong et al., 2003; Zhang et al., 2016). Glucosinolates are biologically active compounds produced by members of the family Brassicaceae and belong to the sulfur and nitrogen group (Cartea and Velasco, 2008; Singh, 2017). Their direct defensive capacity against insect herbivores and also microbes is well recognized (Wittstock et al., 2003; Hopkins et al., 2008). In the absence of an attack, they are produced and stored in an inactive form. Upon attack, they are activated via hydrolysis by the enzyme myrosinase to produce toxic compounds to insect pests such as isothiocyanates (Kim et al., 2008; Bejai et al., 2012). The nitrogen-based metabolites include alkaloids (in 20 % of all plant species), and cyanogenic glucosides. They are recognized to primarily defend plants against pathogens and insect pests (Way, 1984; Friedman, 2002).

The induction of these secondary metabolites has been demonstrated as the first line of defense against different insect herbivores. For example, in *Brassica* spp, the production and activation of glucosinolates occur upon herbivory by chewing and piercing-sucking insects or chemical treatments (Kim et al., 2008; Bejai et al., 2012). This illustrates that glucosinolates play an essential role in defense against insect herbivores (Textor and Gershenzon, 2009; Bakhtiari et al., 2018). Further studies show that mutations in genes that regulate the biosynthesis and activation of glucosinolates can render the plants more susceptible to herbivory, e.g., *Spodoptera littoralis*, mollusks, among other herbivores (Schlaeppi et al., 2008; Falk et al., 2014). Alkaloids, including the pyrrolizidine alkaloid produced by *Senecio jacobaea* and nicotine produced by tobacco (*Nicotiana attenuata*), also increase after attack. Several studies show that the silencing of nicotine in tobacco (*N. attenuata*) results in enhanced performance of *M. sexta, Spodoptera exigua,* and other insect herbivores (Steppuhn et al., 2004; Machado et al., 2016).

The effects of secondary metabolites on piercing-sucking herbivores are less clear. Variable effects of secondary metabolites on aphids feeding have been reported. For instance, bioassays testing the effect

of pyrrolizidine alkaloids on pea aphid revealed mild effects, whereas the same aphids were strongly deterred when offered indolizidine and quinolizidine alkaloids (Dreyer et al., 1985). In another study, the potato aphid *(Macrosiphum euphorbiae)* was deterred when exposed to aglycones of steroidal glycoalkaloids compared to the steroidal glycoalkaloids (Günner *et al.* 1997). Although I highlighted several mechanisms primarily reported as the mediators of direct defenses, much needs to be done to reveal the unidentified novel molecules involved in plant resistance against herbivores and other stresses.

## 1.3 The 'watchdog' role of phytohormones in the activation of plant defense responses

Plant hormones or, 'phytohormones' are natural organic substances that regulate diverse plant physiological events at low concentrations. Such events include plant growth, differentiation, and development, as well as signaling networks that are involved in plant response to different abiotic and biotic stimuli (Hirsch et al., 1997; Adie et al., 2007b; Gutierrez et al., 2009; Pieterse et al., 2009; Wasternack and Hause, 2013). Plant hormones including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are central players in the regulation of induced defense responses to herbivores and pathogens (Bari and Jones, 2009; Pieterse et al., 2009, 2012; Nguyen et al., 2016a).

Salicylic acid (SA) is a phenolic compound with direct and indirect roles in regulating many metabolic and physiological processes in plants (Vlot et al., 2009; Rivas-San Vicente and Plasencia, 2011; Dempsey and Klessig, 2017). Moreover, SA is vital to regulate plant defense responses against biotrophic pathogens, and some insect herbivores (An and Mou, 2011; War et al., 2011; Dempsey and Klessig, 2017; Palmer et al., 2017). The SA-dependent downstream defenses are governed by the transcriptional regulator non-expressor pathogenesis-related gene (*NPR1)*, and eventually, culminating in activation of defense-related genes such as *pathogen-related protein 1 (PR1)* (van Loon et al., 2006; Vos et al., 2015). Exogenous application of SA can affect various physiological, biochemical, and molecular processes such as the expression of *PR* and *PI* genes that increase resistance against pathogens (Doherty et al., 1988; Ward et al., 1991). Experimental evidence shows that the induction of the SA pathway is involved in direct and indirect defenses (Mohase and van der Westhuizen, 2002; James, 2003; de Boer et al., 2004; Lortzing et al., 2019). For instance, in *A. thaliana* oviposition by *P. brassicae* induced SA signaling (Bruessow et al., 2010). The larvae hatching and subsequently feeding on plants with egg induced SA levels gained less weight (Lortzing et al., 2019). Investigation on the indirect effects of SA revealed that it is first modified to form the bioactive SA derivative methyl salicylate (MeSA), which attracts natural enemies to insect herbivores, for example in lima beans, tomato, tobacco, and potato infested with spider mites and beetles (de Boer et al., 2004; Park et al., 2007; Rodriguez-Saona et al., 2011; War et al., 2012; Filgueiras et al., 2019). It is noteworthy to remark that not all plant-herbivore combinations lead to the production of MeSA. For instance, *S. exigua*

feeding on *N. attenuata* induces the synthesis of SA, which activates SA-inducible genes but not MeSA (Diezel et al., 2009).

Jasmonic acid (JA) is a derivative of fatty acid metabolism. Together with the pre-cursor *cis*-(+)-12-*oxo*-phytodienoic acid (OPDA) and conjugate (+)-7-iso-jasmonyl-*L*-isoleucine (JA-Ile), it belongs to the jasmonates (JAs). The JAs play essential roles in regulating defense responses against insect herbivores and microbial pathogens (Liechti and Farmer, 2002; Pozo et al., 2004; Schaller and Stintzi, 2008). Each active form of JAs (OPDA, JA, JA-Ile) and related metabolites, including MeJA and cis-jasmone, play roles in plant physiology and are directly or indirectly involved in plant response to herbivory (Larrieu and Vernoux, 2016; Aljbory and Chen, 2018). The perception of herbivores feeding and pathogen attack can trigger the biosynthesis of JA, and the ecological roles of the induced JA accumulation have been studied in several systems comprising of mutants and wild type plants (Li et al., 2002; Bari and Jones, 2009; War et al., 2012). For example, in tomato mutants compromised in the ability to accumulate JA (*def1*) showed reduced resistance to *M. sexta* caterpillars (Howe et al., 1996). In another study, the western flower thrips (*Frankliniella occidentallis*) oviposited more on *A. thaliana* mutants (JA-insensitive *coi1*-1) compared to wild type (Abe et al., 2009). Also, treatment of mutant plants with exogenous MeJA and JA restored the wild type functions, including expression of defense genes, induction of chemical responses, and other defense traits commonly induced against insect herbivores (Thaler et al., 2001; Halitschke and Baldwin, 2003; Cooper and Goggin, 2005). In the absence of JA, OPDA can regulate plant defense against pathogens and herbivores via induction of *12-oxophytodienoate reductase* (OPR) genes in tomato (Stintzi et al., 2001; Bosch et al., 2014b; Scalschi et al., 2015), *A. thaliana* (Stintzi et al., 2001; Bosch et al., 2014a) and the *Maize insect resistance 1-Cysteine protease* gene in maize (Varsani et al., 2019). In another study, the exogenous application of MeJA on *A. thaliana* resulted in the accumulation of OPDA, hence supporting the role of OPDA in regulating plant defenses (Gleason et al., 2016). Moreover, exogenous treatment of plants with MeJA can activate inducible defense responses similar to those induced during herbivory. For example, in *N. attenuata*, topical application of MeJA induced accumulation of protease inhibitors similar to *M. sexta* caterpillars feeding on the same plant (van Dam et al., 2001a). The JA conjugate JA-Ile also coordinates plant defenses against herbivory. For example, in *N. attenuata*, the silencing of JA hydroxylases increased the JA-Ile levels and the plant's resistance against several insect herbivores: *S. litura, S. littoralis* and *M. sexta* (Woldemariam et al., 2012; Tang et al., 2020). The role of JA-Ile in indirect plant defense has also be studied. Using the model plant *N. attenuata,* it has been demonstrated that JA-Ile regulates specific branches of the JA pathway, leading to the production of volatile secondary metabolomes (Schuman et al., 2018). Further comparisons on the susceptibility level of jasmonate-deficient (*asLOX3*) vs. JA-Ile deficient (*irJAR4xirJAR6*) *N. attenuata* mutants and showed that the JA-Ile deficient plants were better defended than the jasmonate-deficient plants. Additional analysis using JA to elicit the emission of (*E*)-*α*-

bergamotene (herbivore-induced volatile that is regulated by JA), revealed that in the JA-Ile deficient plants the emission of (*E*)-*α*-bergamotene was more compared to the wild types, implying that JA-Ile regulates specific branches of the JA pathway (Schuman et al., 2018). Moreover, the specificity associated with the JA-Ile response might be responsible for governing trade-offs between resistance and other plant processes (Schuman et al., 2018).

Ethylene (ET) is a gaseous plant hormone which influences multiple processes such as leaf and flower development, leaf abscission, fruit ripening, sex determination in plant growth and development (Dolan, 1997; Chang, 2016; Iqbal et al., 2017). Besides, ET plays a role in the activation and signaling of defense responses against a broad range of pathogens and insect herbivores (Ecker and Davis, 1987; Adie et al., 2007b; von Dahl and Baldwin, 2007). However, it is important to remark that, ET can have variable effects in defense regulation because it acts mainly as a modulator of other hormonal pathways rather than a direct elicitor (Adie et al., 2007b). For instance, the ET interaction with the phenylpropanoid pathway results in the production of phytoalexins, and PR proteins as direct defense responses to herbivory (Ecker and Davis, 1987; Bouchez et al., 2007; von Dahl and Baldwin, 2007; Broekgaarden et al., 2015; Pangesti et al., 2016). It is also known that an ET burst can modulate indirect defenses upon plant damage by insects. For instance, insect herbivory, as well as the exogenous application of ET, has been shown to trigger the emission of plant volatiles in several plant species including maize, beans, pine, tomato, onion, among other plants (Horiuchi et al., 2001; von Dahl and Baldwin, 2007).

Abscisic acid (ABA) is synthesized from isoprenoids in the terpenoid synthesis pathway and regulates many aspects of plant growth and development, including seed germination, leaf senescence, adaptation of the plant to abiotic and biotic stresses (Bari and Jones, 2009; Dempsey and Klessig, 2017). Recent studies show that ABA involvement in defense regulation helps the plants to fully activate defense and resistance against herbivorous insects (Thaler and Bostock, 2004; Bodenhausen and Reymond, 2007; Nguyen et al., 2016b). For instance, in several plant species, including *A. thaliana*, tomato, and *N. attenuata,* the decrease in ABA concentration increases plant susceptibility to herbivory (Thaler and Bostock, 2004; Truong et al., 2013; Vos et al., 2013). In bioassays involving ABA-deficient tomato (*sitiens*) and potato (*droopy*) mutants, it was found that exogenous application of ABA restored high levels of *P II* gene leading to a higher herbivory resistance (Pēna-Cortés et al., 1989). In the case of plant-pathogen interactions, ABA activity appears more complex. Indeed, it has been demonstrated that the presence or absence of ABA biosynthesis and perception can, in some cases, increase the plant's resistance (Mohr and Cahill, 2003; Achuo et al., 2006) or susceptibility to pathogens (Adie et al., 2007a). Further studies report that topical treatment of plants with ABA increases susceptibility to attack by pathogenic bacteria and fungi (Koga et al., 2004; de Torres-Zabala et al., 2007). These variations in outcomes are attributable to differences in the systems under investigation and the context-dependence in each system (reviewed by; Bari and Jones 2009).

Besides the direct effects of ABA on resistance, it has been shown that it regulates the stomatal closure to hinder pathogen entry into plant cells. For example, exogenous treatment of *A. thaliana* with ABA increased resistance levels and stomatal closure to hinder infection by pathogenic microbes (Melotto et al., 2006).

Phytohormones do not operate in isolation. They interact with each other either synergistically or antagonistically and establish complex signaling networks that regulate different pathways and metabolic processes essential for plant development and defense responses (Pieterse et al., 2009; Thaler et al., 2012). This process is referred to as "cross-talk"; it allows the plants to save costs by fine-tuning defenses that are more specified to the type of attacker (Pieterse et al., 2009; Thaler et al., 2012; Ohri et al., 2015). Here, I outline the cross-talk between the principal phytohormone pathways. Cross-talk between the JA and SA pathways has been studied extensively in a variety of systems and primarily documented as mutually antagonistic (Pieterse et al., 2009, 2012; Thaler et al., 2012; Li et al., 2019). The inhibitory effects of SA on JA have been demonstrated in several plants where the activation of the SA pathway results in increased plant's susceptibility to attackers that are usually sensitive to the JA pathway (Mur et al., 2006; Li et al., 2019). For example, herbivory by silverleaf whitefly *(Bemisia tabaci* type B*)* on *A. thaliana* triggers SA production locally and systemically hence suppresses the JA dependent responses (Zarate et al., 2007). In *N. attenuata,* herbivory by *S. exigua* amplifies the SA burst, which lowers the JA burst (Diezel et al., 2009). On the other hand, studies show that JA can antagonize the SA pathway. Early studies involving the exogenous application of a high concentration of JA on *A. thaliana* plants revealed the attenuation of the SA pathway (Mur et al., 2006). In *Brassica nigra* the induction of the JA pathway triggered by the root-knot nematode (RKN) *Meloidogyne hapla* antagonized the SA pathway*,* making the plants more attractive for the aphid *Brevicoryne brassicae* (van Dam et al., 2018). Besides the JA-SA antagonism, synergistic JA-SA interactions upon insect-herbivore feeding also occur. However, the JA-SA synergy mainly occurs in situations where both phytohormones are simultaneously induced and produced in low concentrations (Beckers and Spoel, 2006; Mur et al., 2006; Li et al., 2019). The induction of JA and SA signaling occurs with considerable specificity depending on the herbivore's feeding style as well as the system under investigation. Therefore, it can be concluded that cross-talk between the JA and SA pathways might depend on these factors as well (Bari and Jones, 2009). Although the phenomenon of phytohormone cross-talk is well established, the molecular mechanisms through which the antagonism or synergism occurs is debatable. A discussion by Li et al. (2019) shows that the antagonistic effect of the SA-signaling pathway on the JA-signaling pathway occurs downstream of the JA biosynthesis (Spoel et al., 2003; Leon-Reyes et al., 2010). In other cases, the SA signaling pathway antagonizes the JA signaling pathways at the gene transcription levels (Bari and Jones, 2009; Nguyen et al., 2016a; Verma et al., 2016; Li et al., 2019). On the other hand, the negative effect of the JA-signaling pathway on the SA-signaling pathway might occur at the transcription level. For example, the transcription factor WRKY70 of the JA-signaling pathway

antagonizes the SA-related *PR* genes (Li et al., 2004). More studies are needed to elucidate the molecular mechanism underlying cross-talk between these pathways in different plants.

## 1.4 Interactions between aboveground and belowground induced responses in plants

Terrestrial plants interact with numerous herbivores and pathogens that attack the BG and AG compartments. In order to reduce damage, plants induce an array of defense responses that are regulated via phytohormonal pathways. Induced defense responses are expressed locally (at the damaged tissue), and are also signaled to systemic tissues (undamaged plant parts) to protect them from subsequent attack. Although the majority of studies investigate the local and systemic induced plant responses to herbivory in AG and BG compartments in isolation (Suzuki et al., 2004; Dugravot et al., 2007; Babst et al., 2009; Hao et al., 2012), it is known that induced responses can travel across the BG -AG interface to link these compartments as well as the associated interactions. Indeed, several studies conducted since the late 1980s have made the AG-BG plant-mediated interactions an active research area (Gange and Brown, 1989; Bezemer et al., 2003; van Dam and Raaijmakers, 2006; Kutyniok et al., 2014; Hoysted et al., 2018; Machado et al., 2018; Karssemeijer et al., 2020). Herbivory on either compartment can trigger changes in nutrients and chemical defenses in the opposite compartment. As described previously, phytohormones, notably JA and SA pathways, are central players in regulating the induced defense responses to insect herbivores and pathogens. Here I provide an overview of plant-mediated interactions between AG and BG herbivores in the context of changes in chemical defenses and nutritional composition.

Aboveground plant parts can be attacked by insect herbivores of different feeding guilds, including chewing and piercing-sucking insects. Feeding by these AG insect herbivores can, in general, affect plant fitness and also alter the AG and BG chemical defense responses. The systemic induced BG defense responses can influence the root interacting herbivores. For example, herbivory by *P. brassicae* on *B. nigra* L. (Brassicaceae) leaves, induces systemic accumulation of indole glucosinolates that reduces the survival of the root herbivore *Delia radicum* by 50 % (Soler et al., 2007a). In another study, AG herbivory by *M. sexta* on *N. attenuata* increases the abundance of the RKN *M. incognita* in field and greenhouse experiments. This effect is attributed to the systemic induction of JA signaling (Machado et al., 2018). Besides the changes in chemical defenses, AG herbivores also can change the nutrients in BG organs and influence the performance of root-feeding herbivores. For instance, caterpillar feeding on tobacco plants and mechanical damage on the grass species *Holcus lanatus* enhances the performance of root parasitic nematodes by increasing the amount of nitrogen transported to roots (Kaplan et al., 2008b; Wang et al., 2017). Another study showed that infestation by the aphid *Myzus persicae* on potato leaves results in alteration of glucose and fructose exuded by roots, and diminish the hatching of cysts of the cyst nematode (CN) *Globodera pallida* (Hoysted et al., 2018).

In the BG compartment, plants can interact with multiple herbivores. Among them are two major groups, insect herbivores, and root parasitic nematodes. Root herbivory by these organisms can affect their host in terms of growth and development and induction of systemic responses in BG and also in AG parts. If these root-induced responses are systemic, they can influence the performance of AG insect herbivores. For example, potato root tuber damage by the Guatemalan tuber moth *(Tecia solanivora)* reduces the performance of the caterpillars *S. exigua* and *S. frugiperda*. These negative effects are linked to the increased foliar expression of defense and secondary metabolism marker genes, increased concentrations of chlorogenic acid, and the steroidal glycoalkaloids; *α*-solanine and *α*-chaconine (Kumar et al., 2016). Similarly, in *B. nigra*, root herbivory by the root-lesion nematodes *Pratylenchus penetrans* and the larvae of *D. radicum* induce systemic accumulation of glucosinolates and phenolics that decrease the performance of the caterpillar *Pieris rapae* (L) feeding on leaves (van Dam et al., 2005). Root infection by RKNs has been shown to facilitate the performance of chewing herbivores when sharing a host plant, even when shoot defenses are induced. For example, root infection by the RKN *M. incognita* increases the performance of *S. exigua* and *Epitrix* flea beetles despite an increase in the levels of nicotine (Kaplan et al., 2008b, 2009). Depending on the identity of the root infecting nematode, positive and negative effects on AG piercing-sucking herbivores can occur. For example, root infection by the RKN *M. hapla and M. incognita* increases the preference of the aphid *B. brassicae* in *B. nigra* plants (Hol et al., 2016; van Dam et al., 2018). However, when the same aphid species is feeding on the *Brassica* plants *(B. nigra* and *B. oleracea)* infested by CN *Heterodera schachtii* they show reduced preference and longer time to reproduction (Hol et al., 2013; van Dam et al., 2018). Belowground herbivores can affect the nutrient quality in AG plant organs and consequently affect the AG associated insect herbivores. For example, root infection by the RKN *M. incognita* in *N. tabacum* increases the levels of invertase enzyme in the giant cells indicating a strong sink for plant resources and consequently decreases by 55 % the growth and fecundity of the aphid *M. persicae* (Kaplan et al., 2011). Similarly, when the same aphid species infested *Beta vulgaris* L with prior root infection by the CN *H. schachtii,* the performance decreases due to the reduction in the amounts of sugars in leaves (Hol et al., 2010). Based on these studies, it is tempting to conclude that, generally, root parasitic nematodes mainly affect aphids negatively. However, there can be exceptions. For instance, root infection by the RKN *M. hapla* results in increased sugar levels in *B. nigra* leaves that increase the population of the aphid *B. brassicae* (Hol et al., 2016). Therefore, more studies on a case by case basis are required to shed more details on the dynamics of parasitic nematode -plant-aphid interactions.

In summary, studies investigating systemic induced defense responses triggered after AG and BG herbivory show differential effects on herbivores in the opposite compartment. Such differential outcomes of AG-BG interactions can be attributed to several factors including differences in the feeding style of each herbivore, host plant susceptibility level, identity of the attacking herbivore, temporal variation, and

experimental designs (Kaplan et al., 2008b; Olson et al., 2008; Erb et al., 2011; Wondafrash et al., 2013; Heinen et al., 2018; van Dam et al., 2018). Although these factors are mostly deduced from studies involving insect herbivores, some of them are relevant in plant-mediated interaction between root-feeding parasitic nematodes such as the RKNs and AG insect herbivores including caterpillars and piercing-sucking insects which form the basis of this thesis.

Root parasitic nematodes such as RKNs are dominant soil residing pathogens that infect the roots of numerous plants and establish feeding sites by reprograming both the plant's developmental and defense pathways. It has been demonstrated that these local changes can interfere with the distribution of resources and defense status of the plant (Yamaguchi et al., 2017; Gheysen and Mitchum, 2019). A growing body of studies shows that the locally induced changes in defenses and nutritional composition do not only occur in roots but also at distal undamaged tissues such as AG organs (Kaplan et al., 2008a, 2011; Guo and Ge, 2017). In return, these systemic changes can affect the plant interactions with AG insect herbivores. The resultant ecological effects on the AG insect herbivores are diverse, ranging from positive (Kaplan et al., 2008b, 2009; Hoysted et al., 2017; van Dam et al., 2018) to negative (Kaplan et al., 2011; Arce et al., 2017; Guo and Ge, 2017; van Dam et al., 2018). Although virtually not studied, it has also been shown that AG insect herbivores feeding on RKN infected plants can have reciprocal effects on the root infecting parasitic nematode such as the RKNs (McCarville et al., 2012). Despite the increase in data and information on these interactions, the underlying patterns are difficult to identify due to a high degree of context-dependency. Indeed, the AG-BG plant-mediated interactions involving the RKNs are complex because the nematode establishes a long-term intimate association with the host plant throughout the nematodes' life cycle. Following this line, the reprogramming of plant developmental and immunity systems might change throughout the RKN's life cycle. Moreover, the effects of RKN on AG defenses responses and insect herbivores performance as well as the reciprocal effects of feeding by AG insect herbivores on the RKN and the root defense responses might change as a function of the RKNs' life cycle. In this thesis, I investigated the interaction between RKN and AG caterpillars and aphids at different stages of the nematodes' infection cycle.

## 1.5 Research objectives

The main focus of this thesis is to decipher the key elements of the molecular and chemical mechanisms of plant defense responses to sequential belowground infection by root parasitic nematodes (RKNs) and aboveground insect herbivory. I tested the general hypothesis that the effect of root infection by the RKN *Meloidogyne incognita* on AG plant defense responses and the insect herbivores (caterpillar: *Spodoptera exigua,* and aphid: *Macrosiphum euphorbiae*), and the reciprocal effect of the AG insect herbivores on root defense responses triggered by the RKN, depend on the specific stage of the nematodes' infection cycle.

**The specific research objectives include:**

1) To investigate the impact of root infection by the RKN *Meloidogyne incognita* on an AG chewing herbivore (*Spodoptera exigua*) during the entire nematode infection cycle (Chapter 2).

2) To investigate whether the impact of an AG chewing herbivore (*Spodoptera exigua*) on the RKN *Meloidogyne incognita* induced defense responses in roots depends on the nematodes' infection cycle stage (Chapter 3).

3) To investigate the impact of root infection by the RKN *Meloidogyne incognita* on an AG phloem-feeding herbivore (*Macrosiphum euphorbiae*) and *vice-versa*, during the entire nematodes' infection cycle (Chapter 4).

## 1.6 Experimental study system

The primary investigation system used in this thesis (Chapters 2 - 4) focuses on the commonly cultivated vegetable crop tomato and some of its most important pests. I selected the root-knot nematode as root herbivore and two insect herbivores, a caterpillar, and an aphid, as leaf feeding herbivores (Figure 2).

### 1.6.1 Tomato (*Solanum lycopersicum*); origin, production, and common pests

Tomato (*Solanum lycopersicum* L.) (Figure 2-the plant) is native to Western South America. It is the second most important vegetable after potato worldwide (El-Sappah et al., 2019). Tomatoes are an essential ingredient in a large variety of raw, cooked, or processed foods (OECD, 2017). Given its value as a food source, tomato has been bred to increase productivity, fruit quality, and resistance to insect-pest and pathogens (Bai and Lindhout, 2007; Kimura and Sinha, 2008). Globally, there are approximately 7,500 varieties grown for various purposes for local use or as an export product. The current production is estimated at 180 million tons of fresh tomato fruit on about 5 million hectares worldwide, with a market value of US$ 190.4 million (Oishimaya, 2017).

Tomato suffers damage from a wide range of insect pests and pathogens. Among them are the RKNs that damage the roots, and herbivorous insects, such as caterpillars and aphids that feed on leaves. Insect pests can reduce the growth, quality, and quantity of tomato yields either directly via feeding or indirectly by transmitting disease-causing pathogens (Baranyovits, 1973; Feng et al., 2003; Guerrieri and Digilio, 2008; Zheng et al., 2011). To reduce these adverse effects of insect pests and pathogens, the management of tomato pests mainly involves the use of chemical or synthetic pesticides. Although these chemicals can be effective, some are banned due to detrimental effects on the environment and human health.

Tomato possesses a natural ability to defend itself actively against insect pests and pathogens. It has been established that herbivory activates phytohormonal signaling in roots and leaves (Seiml-Buchinger et al., 2019). Induction of defense genes expressed as a result of phytohormone biosynthesis and signaling, including *PR* genes and *Meloidogyne incognita-1 (Mi-1)* and other *Mi* genes, have also been reported (Ho et al., 1992; De Ilarduya et al., 2001; Molinari et al., 2014). Furthermore, herbivory-induced production of defense proteins, such as PIs, and secondary metabolites in tomato has been documented (Fontaine and Irving Jr, 1948; Friedman, 2002).

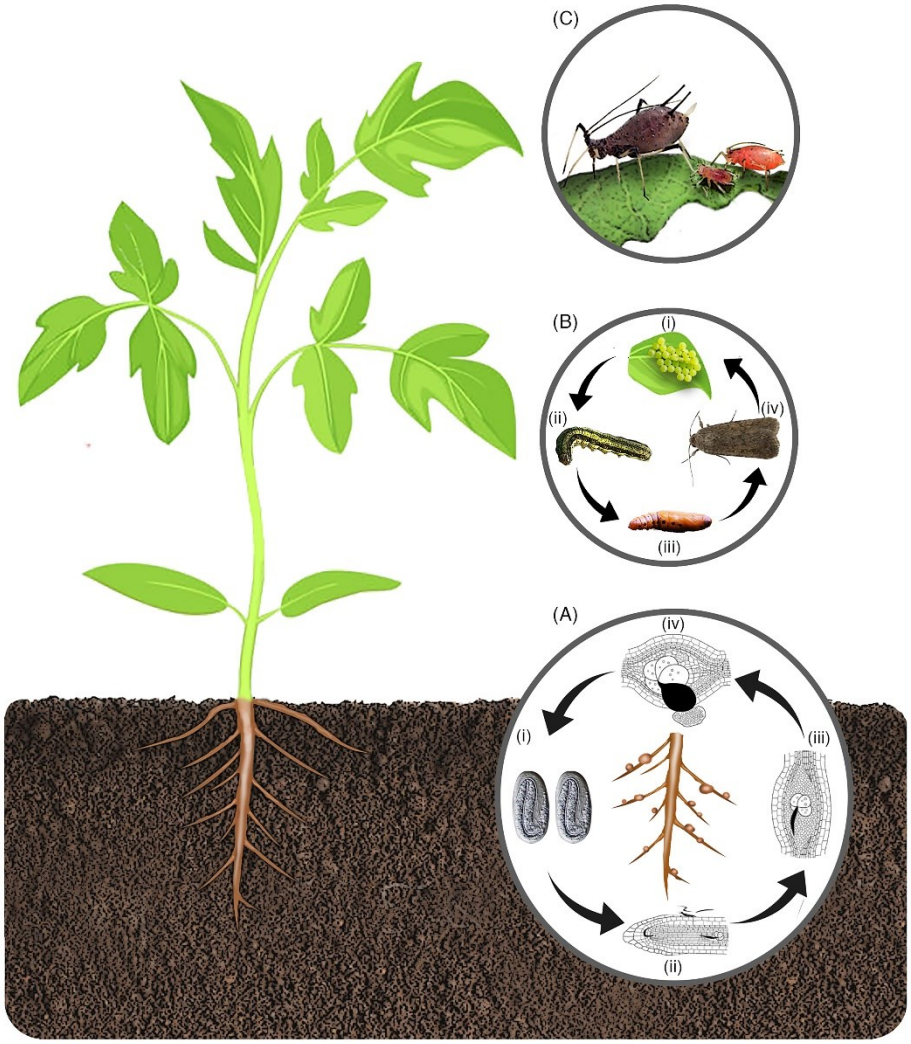


**Figure 2 Schematic diagram of the experimental system**. The experimental system includes the model plant tomato (*Solanum lycopersicum* cv. 'Moneymaker'), and roots and shoots feeding herbivores. The root herbivore is a root-knot nematode (RKN) *Meloidogyne incognita* (A). The RKN is an obligate root feeder, which enters the root and establishes feeding sites in the vascular tissues. Its life cycle can be divided into four main stages; A-(i) egg stage (here cells that develop into infective stage one juveniles (J1) and infective stage two juveniles (J2s) inside the egg). A-(ii) root infection stage (the J2s move out of the eggshell and penetrate the root at the zone of elongation, migrate towards the tip, and turns around to enter the vascular cylinder. A-(iii) establishment stage (the J2 induces several giant cells from which they feed on, and by the third day post-infection, the giant cells expand to form the so-called root knots/galls. A-(iv) reproduction stage (the juveniles gradually molt twice and develops into a female while giant cells and root galls enlarge, and eventually, the pear-shaped mature female lays an egg mass that protrudes from the root surface). The shoot herbivores are (B) *Spodoptera exigua,* which is chewing insect herbivore. The *S. exigua* life cycle includes four main stages B-(i) eggs, B-(ii) larval: five molting stages, B-(iii) pupal, and B-(iv) moth, and (C) *Macrosiphum euphorbiae,* which is a piercing-sucking insect herbivore. The *M. euphorbiae* reproduce parthenogenically, and the nymphs undergo four nymphal stages to reach adulthood. Jennifer Gabriel did the artwork of the figure. Root images modified from Escobar et al. (2015).

### 1.6.2 Root-knot nematode *(Meloidogyne incognita*); life history and pest status

Root-knot nematodes belong to the genus *Meloidogyne*, which includes nearly ~100 nominally described species. The species *M. incognita* selected as the root herbivore for this project (Figure 2A) is an extremely devastating parasite to crops across the globe (Jones et al., 2013). The *M. incognita* is capable of infecting more than 2000 species of higher plants. In most crops, its infection results in reduced plant growth and yield loss valued at more than US$ 125 billion worldwide (Chitwood, 2003). A typical RKN life cycle from eggs to adult consists of four molts or juvenile stages. One complete cycle can take 3-4 weeks, depending on temperature, soil moisture, and the host plant species, among other factors (Williamson and Hussey, 1996; Walker et al., 2003; Badri and Vivanco, 2009). The adult females deposit eggs in a gelatinous matrix on the root surface. The first stage juveniles (J1s) develop inside the egg into the second-stage juveniles (J2s). The J2s are mobile and can infect a susceptible host plant. To locate and infect a susceptible host, the J2s rely on cues such as amino acids and sugars exuded by roots, or chemical cues such as $CO_2$ (Robinson, 2002; Perry, 2005; Čepulytė et al., 2018; Oota et al., 2019). Root penetration occurs near the zone of elongation and is followed by a non-destructive intercellular migration towards the root tip. Then the J2s turn around past the Casparian strip to enter into the vascular cylinder (Fenoll et al., 1997; Perry et al., 2009). They select several vascular phloem cells to induce giant cells from which they feed. The giant cells occur as a result of repeated rounds of nuclear divisions that culminate into multinucleate (>100 nuclei) hypertrophied cells. Further development of these hypertrophied cells forms visible spherical structures commonly called 'gall' or 'root-knot'. The J2s become sedentary and molt twice as J3 and J4 to become adult males or females. It is important to remark that J2s do not possess reproductive organs. However, they differentiate into males or females at the J3 stage, an activity-dependent on the availability of food resources (Abad and Williamson, 2010). The vermiform males are mostly non-feeding and migrate out of the roots, whereas the females remain sedentary and are pear to globose in shape. Females reproduce parthenogenetically and deposit eggs on the root cortex in a gelatinous sac. The sac serves to keep the eggs in a dormant stage until there is a suitable cue for hatching (Williamson and Hussey, 1996; Abad and Williamson, 2010).

As mentioned above, tomato possesses a natural ability to protect itself. This is mostly mediated by phytohormonal signaling. In the case of RKN infection, both JA and SA pathways have been demonstrated to be induced against the nematodes (Cooper et al., 2005; LiNing et al., 2011; Zinov'eva et al., 2013; Molinari et al., 2014). For example, root infection by the RKN increases endogenous JA levels and the expression of *PI II,* as well as SA signaling markers *PR-1* and *PR-5* genes in tomato roots (Sanz-Alférez et al., 2008; Fan et al., 2014; Molinari et al., 2014; Seiml-Buchinger et al., 2019). Despite the induced responses, the RKN still infects the tomato plants. It has been demonstrated that the RKNs utilizes effector proteins to modulate the host plant immunity (Mejias et al., 2019; Vieira and Gleason, 2019).

Secreted effector proteins target a variety of phytohormone receptors, transcriptional activators, repressors, and other components of phytohormone signaling to suppress or neutralize the strength of induced response. For example, in other *Solanum* species such as *Nicotiana bethamiana* the RKN *M. incognita* secretes the effector chorismate mutase, which lowers the SA levels (Haegeman et al., 2012; Wang et al., 2018a). The modulation of root defenses by RKN can result in systemic induction in AG plant organs. Changes in the expression of defenses in AG tissues of RKN infected tomato have been reported (Molinari et al., 2014). Generally, RKN-induced systemic responses in shoots lead to an increase in the performance of chewing herbivores and negative effects on phloem feeders (Wondafrash et al., 2013; Biere and Goverse, 2016).

### 1.6.3 *Spodoptera exigua;* life history and pest status

*Spodoptera exigua* (Hübner 1876, Lepidoptera: Noctuidae), also known as the beet armyworm (BAW), was selected as the leaf chewer for this research project (Figure 2B). The BAW originated from Southeast Asia and has expanded its geographical range globally due to increased trade and travel (FAO: www.fao.org/news/story/en/item/1187738/icode/). It has a host range of more than 50 plant species distributed across 10 plant families worldwide, including *Solanum* species such as potato and tomato (Greenberg et al., 2001). The female moths lay eggs in batches of 50-150 on the lower surface of their host plant leaves. The emerging/hatching larvae develop through five molting stages. The first instars are gregarious, whereas, from the second instar, they become solitary and disperse over the whole plant. They mainly feed at night (hence the name noctuid) and hide during the day. The mature larvae (fifth instar) drop to the ground, dig an underground silk woven chamber where they pupate (Capinera, 2017).

In agro-ecosystems, BAW is considered amongst the most notorious pests worldwide. This is because BAW has a broad host range and can spread very rapidly. In order to spread, the moths fly to an elevation of 200-500 m high and gain wind assistance to travel over long distances (approximately 179 km) (Feng et al., 2003; Zheng et al., 2011). In tomato crops, infestation by *S. exigua* in the early growing period is more damaging than when the plants are more grown. Indeed as few as one caterpillar per 20 tomato plants can reduce plant's fitness and growth and cause economic loss in the agro-industry (Taylor and Riley, 2008; Capinera, 2017).

Like other chewing herbivores, BAW caterpillars trigger JA-dependent responses in plants. In tomato plants, JA-induced responses, including OPDA, JA, and JA-Ile and defense proteins such as polyphenol oxidase, PIs, cause direct negative effects on the survival of BAW (Constabel et al., 1995; Thaler et al., 2002a; Bhonwong et al., 2009; Bosch et al., 2014b, 2014a). On the other hand, *S. exigua* can overcome the induced responses by exploiting mid-gut microbes, and inducing SA- responses to attenuate the effect of the JAs via cross-talk (Thaler et al., 1999, 2002b; Diezel et al., 2009). As a result of the negative SA-JA cross-talk, the *S. exigua* larvae performance on the plants is enhanced. Besides cross-talk, BAW

secretes catalases in salivary fluid during feeding to reduce the formation of hydrogen peroxide as an elicitor of inducible plant defenses against them (Felton and Duffey, 1991).

### 1.6.4 *Macrosiphum euphorbiae;* life history and pest status

*Macrosiphum euphorbiae* (Thomas 1878, Hemiptera: Aphididae), the potato aphid, was also selected as the sap-sucking herbivore for this research project (Figure 2C). The potato aphid originated in North America, and now it is a cosmopolitan pest with global distribution. As a polyphagous herbivore, it can colonize over 200 plant species belonging to 20 families, including *Solanum* species (Saguez et al., 2013). Phenotypically their body color appears yellowish-green to pinkish-red. They display a dimorphism and switch between winged (alate) and wingless (apterous) forms. The wing dimorphism depends on the availability of resources, where the apterous form increases in frequency in the population when there are enough resources (Capinera, 2008; Saguez et al., 2013). Their life cycle consists of four molting stages through which nymphs reach the adult stage. A complete cycle takes 10-30 days. The exact length depends on the quality of host or habitat and present environmental factors.

*Macrosiphum euphorbiae* is ranked among the most damaging insects to agriculture in the world (van Emden and Harrington, 2007). They can build to high population densities within a short time and remove large amounts of plant nutrients by siphoning the phloem sap (van Emden and Harrington, 2007; Flint, 2013). They also excrete honeydew, which can build up and form sooty molds that hinder photosynthesis and promote other fungal diseases (Chomnunti et al., 2014). They inject salivary secretions that are phytotoxic and cause stunting and leaf deformation. However, the most severe problem caused by *M. euphorbiae* is the transmission of plant viruses. The viruses infected plants show yellowing and increased free amino acids that make up nutrition for the aphid (Sorensen, 2009; Kroschel et al., 2020). By combining these effects, *M. euphorbiae* can cause up to 100 % yield loss (Sorensen, 2009). The development of alates under resource limitation enables the quick distribution of the aphid. During flight, the aphid visually identifies a potential host and recognizes an attractive or repulsive cue via olfaction (Powell et al., 2006). Upon landing on plants, it utilizes its antennae to detect odors and the tarsal contact to explore the surface texture (Pettersson et al., 2007). By probing tissues with their stylets, they assess the host plant for the presence of phytochemicals and other anti-herbivore components.

Although aphids are 'stealthy feeders' (Voelckel et al., 2004) that cause minimal damage to plant tissues, they can trigger defense responses. In tomato plants, hormonal signaling is initiated upon recognition of aphid associated molecular patterns/effectors by plant receptors. For example, induction of SA and the increase in related genes enhances resistance to the potato aphid (de Ilarduya et al., 2003; Li et al., 2006). To counter the induced defense responses, the potato aphid utilizes salivary proteins secreted during feeding to reduce the strength of plant responses. For example, the application of the effector protein

*Me47* (characterized from the aphid *M. euphorbiae*), on tomato leaves results in enhanced fecundity of the aphid *M. euphorbiae* (Kettles and Kaloshian, 2016). In addition, JA-dependent responses are also linked to resistance against *M. euphorbiae*. Induction of *Lox* and repression of *PIs* genes by the potato aphid in tomato leaves correspond to its enhanced herbivory (Fidantsef et al., 1999; Cooper and Goggin, 2005).

## ACKNOWLEDGMENTS

**Crispus M. Mbaluto** (CMM) made an outline for the thesis introduction, which was reviewed by and discussed with **Nicole M. van Dam** (NMvD). Based on outline plus comments, CMM produced a first full version of the introduction, which was reviewed, commented, and partially corrected by NMvD and **Ainhoa Martínez-Medina**. CMM produced the final version of the introduction. CMM acknowledges **Jessil Ann Pajar** for proofreading the phytohormone section and **Jennifer Gabriel** for the artwork of the figures.

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# OUTLINE OF MANUSCRIPTS

## Chapter 2

### The impact of *Meloidogyne incognita* root infection on *Spodoptera exigua* varies over the nematodes' infection cycle

Crispus M. Mbaluto, Fredd Vergara, Nicole M. van Dam, Ainhoa Martínez-Medina
(Submitted on/under review (30-05-2020) in Journal of Experimental Botany)

In chapter 2, I investigated whether the impact of root infection by the root-knot nematode (RKN) *Meloidogyne incognita* on aboveground caterpillar *Spodoptera exigua* is modulated by the nematodes' infection cycle. To achieve this objective, I conducted a series of experiments in which the RKN infected plants first and at 5, 15, and 30 days post-infection (dpi) corresponding to the nematodes' invasion, galling, and reproduction stages, each shoot was infested with a second-instar caterpillar for 24 h. I showed that root infection by the *M. incognita* enhanced the performance of *S. exigua* only during the nematodes' galling stage. To understand the main mechanisms driving this effect, I analyzed the systemic impact of the RKN on the profile of jasmonates, including 12-*oxo*-phytodienoic acid, jasmonic acid, and jasmonyl-*L*-isoleucine, trypsin protease inhibitors and related marker genes, and defense metabolites triggered aboveground by *S. exigua*. The results obtained revealed that *M. incognita* root infection affected systemically the jasmonates-related response and metabolites profiles triggered aboveground by *S. exigua* feeding, and that this effect was modulated during the nematodes' infection cycle. Collectively, this study demonstrates that the impact of RKN root infection on aboveground defense responses and the performance of chewing herbivores depends on the nematodes' infection cycle.

**Crispus M. Mbaluto** contributed to the conception of the idea, designed and carried out the experiments, processed samples, data analysis, and interpretation, writing the initial manuscript. **Fredd Vergara** contributed by reviewing the chemistry section in the manuscript, interpretation of metabolomics data, and preparation of figures for the chemistry section; **Nicole M. van Dam** and **Ainhoa Martínez-Medina** contributed in the conception of the idea, supervision of the work, reviewing the manuscript and final approval for submission.

## Chapter 3

### The impact of *Spodoptera exigua* herbivory on *Meloidogyne incognita* induced root responses depends on nematodes' life cycle stage

Crispus M. Mbaluto, Esraa M. Ahmad, Melody Fu, Ainhoa Martínez-Medina, Nicole M. van Dam

**Published in Annals of Botany Plants (AoBP) DOI: 10.1093/aobpla/plaa029 (2020)**

In chapter 3, I investigated whether the impact of aboveground herbivory on root defense responses induced by the root-knot nematode (RKN) *Meloidogyne incognita* depends on the nematodes' infection cycle stages. To achieve this objective, I conducted a set of experiments in which the RKN was infected first, and at 5, 15, and 30 days post-infection (dpi) corresponding to nematodes' invasion, galling and reproduction stages each shoot was infested with a second-instar *Spodoptera exigua* caterpillar for 24 h. I analyzed changes in the phytohormones: jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), and the main steroidal glycoalkaloid in tomato, *α*-tomatine. In addition, I examined the expression of defense-related and glycoalkaloid metabolism (GAME) genes. I showed that, nematode infection alone increased the endogenous root levels of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), *α*-tomatine, and the expression of the *GLYCOALKALOID METABOLISM 1 (GAME1)* gene mostly at 30 days post nematode inoculation. Caterpillar feeding alone upregulated *Lipoxygenase D* and downregulated *Basic-β-1-glucanase* and *GAME1* expression in roots. On nematode-infected plants, caterpillar feeding decreased JA levels, but it increased the expression of *Leucine aminopeptidase A*. The induction patterns of ABA and SA suggest that caterpillars cause cross-talk between the JA-signaling pathway and the SA and ABA pathways. Our results show that caterpillar feeding attenuated the induction of the JA pathway triggered by nematodes, mostly in the nematodes' reproduction stage. These results generate a better understanding of the molecular and chemical mechanisms underlying frequent nematode-plant-caterpillar interactions in natural and agricultural ecosystems

**Crispus M. Mbaluto** contributed in the conception of the idea, experimental design, conducting the experiments, processing of samples, data analysis and writing of the initial manuscript; **Esraa M. Ahmad** and **Melody Fu** contributed in the processing of samples and data analysis, and reviewing the manuscript; **Ainhoa Martínez-Medina** and **Nicole M. van Dam** contributed in the conception of the idea and experimental design, supervision, critical revision of the manuscript and approval of the final manuscript for submission.

## Chapter 4

### Induced local and systemic defense responses in tomato to mediate the interaction between root-knot nematode and potato aphid

Crispus M. Mbaluto, Nicole M. van Dam, Ainhoa Martínez-Medina
(Manuscript in preparation)

In chapter 4, I Investigated how the root infection by the RKN *Meloidogyne incognita* affects shoot responses triggered by the aphid *Macrosiphum euphorbiae*, and the concomitant impact of shoot herbivory by *Ma. euphorbiae* on root responses to *M. incognita* infection. Moreover, as in the previous chapters I found that plant responses to *M. incognita* are tightly modulated throughout the infection cycle, I further studied the dynamic of the plant responses to the reciprocal interaction between *M. incognita* and *Ma. euphorbiae* during the entire nematode infection cycle. I followed a similar experimental set up as in the above chapters 2 and 3. But here, each plant shoot was infested with 12 individuals of the aphid for 24 h. I analyzed leaf and root levels of the phytohormones jasmonic acid (JA), JA-isoleucine (JA-Ile), salicylic acid (SA), abscisic acid (ABA), and auxin (indole-3 acetic acid: IAA), as well as the expression dynamics of marker genes in the JA (*proteinase inhibitor II*, *PI-II*) and SA (*pathogen-related protein 1*, *PR1*) pathways. Also, we measured the levels of the steroidal glycoalkaloids (SGAs) *α*-dehydrotomatine and *α*-tomatine and the expression of glycoalkaloid metabolism (GAME) genes; *jasmonate-responsive ETHYLENE RESPONSE FACTOR (ERF) transcriptional factor 4* (*JRE4*) and glycoalkaloid metabolism 1 (*GAME1*). I showed that that aphid feeding neither affected the levels of phytohormones nor the expression of *PI-II* and *PR1* in leaves. However, SGAs levels and the expression of GAME genes decreased in leaves in 4.5-6 weeks old plants infested with aphids only. In the roots, aphid feeding decreased the levels of JA, ABA, and IAA, but only in plants that were 8 weeks old. Aphid's feeding neither changed SGAs levels nor the expression of GAME genes systemically in roots. Nematode root infection increased root SA levels throughout the infection cycle and ABA levels only at the reproduction stage. Levels of SGAs and the expression of GAMEs increased locally at the galling stage. Leaf SA levels increased in plants where RKNs were at the reproduction stage. SGA levels and the expression of GAME genes decreased in leaves of plants with RKNs at the invasion stage. Aphid feeding on nematode-infected plants did not alter the systemic effects of nematodes on SA showed only mild effects on the leaf phytohormone and SGA levels, and the GAME pathway. In roots of double infested plants, root JA-Ile levels were higher when RKNs were in the galling stage. In the same plants, *PI II* expression, a marker for the JA pathway, was lower in roots. These differences did not translate in differences in GAME expression or SGAs in roots between plants with only RKN in the galling stage and double infested plants. In none of the other developmental stages, there was an interactive effect of aphid feeding on nematode-induced roots responses. This means that, overall, nematode feeding had a stronger effect on AG aphid-induced responses, than the reverse. The stage of the RKN nematode co-determined the strength of the effect

**Crispus M. Mbaluto** contributed to the conception of the idea, designed and carried out the experiments, processed samples, data analysis, and interpretation, writing the initial manuscript. **Nicole M. van Dam** and **Ainhoa Martínez-Medina** contributed to the conception of the idea, supervision of the work, reviewing the manuscript, and final approval for submission.

# CHAPTER 2

# The impact of *Meloidogyne incognita* root infection on *Spodoptera exigua* varies over the nematodes' infection cycle

# The impact of *Meloidogyne incognita* root infection on *Spodoptera exigua* varies over the nematodes' infection cycle

Crispus M. Mbaluto[1,2], Fredd Vergara[1,2], Nicole M. van Dam[1,2], Ainhoa Martínez-Medina[1,2,3]

[1]Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig; Leipzig, Deutscher Platz 5e, 04103, Leipzig, Germany; [2]Institute of Biodiversity, Friedrich-Schiller-Universität-Jena; Dornburger Straße 159, 07743 Jena, Germany; [3]Plant-Microorganism Interaction Unit, Institute of Natural Resources and Agrobiology of Salamanca (IRNASA-CSIC), Cordel de Merinas, 40, 37008, Salamanca, Spain

**Submitted/Under review 30-05-2020 in Journal of Experimental Botany**

## ABSTRACT

Studies on plant-mediated interactions between parasitic root nematodes and shoot herbivores are rapidly increasing. However, the outcomes for the interacting organisms vary, and the mechanisms involved remain ambiguous. We hypothesized that the impact of root infection by the nematode *Meloidogyne incognita* on the performance of the shoot caterpillar *Spodoptera exigua* is modulated by the nematodes' infection cycle. To test our hypothesis, we challenged nematode infected plants with caterpillars when the nematodes' infection was at invasion, galling, and reproduction stages. We found that nematode root infection facilitated caterpillar performance, particularly during the galling stage. To elucidate the mechanisms underlying this effect, we combined molecular and chemical analyses with performance bioassays. Nematode root infection had a minor direct impact on the shoot level of jasmonates, jasmonate-related marker genes, and shoot metabolome. However, nematode infection significantly affected the shoot jasmonate-related responses, and the metabolic profiles triggered by *S. exigua* feeding. The impact of nematode on *S. exigua*-triggered shoot responses varied over the nematode infection cycle. A stronger impact of nematode infection was found when the nematode was at the galling stage. Our results demonstrate that the nematode infection cycle is a critical factor influencing plant-mediated interactions between parasitic root nematodes and shoot herbivores.

**Keywords:** aboveground-belowground interactions, jasmonates, *Meloidogyne incognita*, phytohormones, plant-mediated interactions, untargeted metabolomics, root-knot nematode, *Spodoptera exigua.*

## INTRODUCTION

In natural and agricultural ecosystems, plants are constantly interacting with a multitude of organisms that attack the roots and the shoots. To counteract the attack by enemies, plants possess a sophisticated immune system that recognizes non-self-molecules or signals from their own injured cells (Duran-Flores and Heil, 2016). They respond by activating an immune response against the invader encountered (Pieterse et al., 2012). Plant hormones such as jasmonic acid (JA) and its derivates (jasmonates, JAs), salicylic acid (SA), ethylene, and abscisic acid are central players in the regulation of the plant immune network (Pieterse et al., 2009). After the attack by enemies, induced defenses are usually expressed not only at the damaged tissue, but also systemically in non-attacked plant parts (Biere and Goverse, 2016). Such systemic response enables the plant to protect undamaged tissues and can affect the performance of other organisms feeding on the same plant (van Dam et al., 2005; Hol et al., 2013; Arce et al., 2017; Hoysted et al., 2017). Besides changes in plant immunity, plant interactions with pathogens and herbivorous insects can affect the plant's nutritional status and nutrient allocation patterns. Such changes in primary plant metabolism can have profound consequences on the performance of herbivores feeding on the same plant (Berenbaum, 1995). As a consequence, plants are essential mediators of interactions between organisms that rarely come into direct physical contact with one another (Soler et al., 2013).

Indeed, previous studies demonstrated that belowground (BG) organisms that closely associate with plant roots influence primary and secondary metabolism in aboveground (AG) plant parts, affecting the growth and development of herbivores feeding on AG tissues (Erb et al., 2009; Kumar et al., 2016; Arce et al., 2017; Hoysted et al., 2017; van Dam et al., 2018). These studies report both positive and negative impacts of BG organisms on AG herbivores, depending on the study systems. For instance, aphids preferred *Meloidogyne hapla* root-infected *Brassica nigra* plants, and *Globodera pallida* root-infected potato plants over non-infected plants (Hoysted et al., 2017; van Dam et al., 2018). By contrast, root infection by *Meloidogyne incognita* reduced both oviposition and performance of the leaf miner *Tuta absoluta* in tomato plants (Arce et al., 2017). Similarly, root damage by western corn rootworm *(Diabrotica virgifera virgifera)* reduced the growth of *Spodoptera littoralis* in maize (Erb et al., 2009). An increasing number of studies are trying to disentangle the main mechanisms underpinning the impact of BG herbivores on herbivorous insects feeding on AG tissues (Bezemer et al., 2005; Soler et al., 2007b; Kaplan et al., 2009; Arce et al., 2017; Hoysted et al., 2017; van Dam et al., 2018). However, this information is still very fragmented. For instance, root damage by some herbivores triggers the production of chlorogenic acid in shoots. The produced chlorogenic acid can differentially affect the AG herbivores by being beneficial to some and harmful to others (Johnson and Felton, 2001; Beninger et al., 2004; Erb et al., 2009; Kumar et al., 2016). Similarly, root infection by cyst nematodes induces systemic changes in JA, SA, and auxins in shoots that favor the growth of thrips *(Frankliniella occidentalis)* but deter spider mites *(Tetranychus urticae)* in

*Arabidopsis thaliana* (Kammerhofer et al., 2015a). Moreover, the studies uncovering the main mechanisms driving AG-BG interactions involving nematodes are uncommon (Wondafrash et al., 2013).

Root-knot nematodes (RKNs) are tiny multicellular organisms that parasitize root systems of nearly all plants. They reprogram plant processes in roots to ensure a continuous supply of resources (Gheysen and Mitchum, 2011). The RKN infection cycle consists of different stages, including the invasion of the host roots, followed by the establishment in the root tissues, and reproduction. Once the infective juveniles hatch, they pierce and penetrate the roots at the zone of elongation. They migrate downwards to the root tip where they enter into the vascular cylinder. There they turn and then move upwards to the differentiation zone (Escobar *et al.*, 2015). They select up to eight cells on which they induce their feeding sites, by pumping stylet secretions to cause hyperplasia in the surrounding cells (Caillaud et al., 2008). This entire process results in the formation of visible structures called root knots or galls (Escobar et al., 2015). Several studies demonstrated that plant response directed against the infecting RKNs change during the infection stages. Generally, plant genes associated with secondary metabolism, signal transduction, and defenses are upregulated at the onset of the nematode infection (Puthoff et al., 2003; Alkharouf et al., 2006; Mazarei et al., 2011). At later stages of root infection, repression of genes encoding peroxidases, major intrinsic proteins, and glucose have been documented (Szakasits et al., 2009; Portillo et al., 2013; Afifah et al., 2019). Noticeably, some of these defense responses can also occur systemically in AG organs of plants infected by the RKN in roots (Hamamouch et al., 2011; Kyndt et al., 2014). For instance, in *A. thaliana, M. incognita* root infection triggers the expression of SA and JA related genes in roots but suppresses them in shoots (Hamamouch et al., 2011). Besides the modulation of AG defense responses, several studies show that some of the changes in primary plant metabolism triggered by RKNs are not restricted to the roots, and can also be expressed in AG tissues (Kyndt et al., 2014). The systemic impact of parasitic root nematodes on defenses and primary metabolism has been associated with changes in the performance of herbivores feeding on AG plant parts (Hol et al., 2013; Arce et al., 2017; Hoysted et al., 2017). However, the outcomes of the interactions are variable; positive, negative, as well as neutral effects of parasitic root nematodes on AG herbivores have been reported. For example, *M. incognita* root infection on tobacco plants had no effects on *Manduca sexta*, but it enhanced the performance of *S. exigua* (Kaplan et al., 2009). In another study, inconsistent effects were recorded on *Myzus persicae* populations when feeding on *M. incognita* infected tobacco plants (Kaplan et al., 2009).

As mentioned above, the infection cycle of RKNs is dynamic, and plant responses change significantly over the nematodes' infection cycle. Here we hypothesize that the impact of root infection by RKN on shoot herbivores depends on the stage of the nematodes' infection cycle. By using tomato as a model plant, and by performing a series of bioassays, we analyzed the influence of root infection by the RKN *M. incognita*, at the invasion, galling, and reproduction stages, on plant interactions with leaf feeding

*S. exigua* caterpillars. Our results show that root infection by *M. incognita* facilitates *S. exigua* performance, specifically during the nematodes' galling stage. By performing molecular biology and chemical analyses, we further explored the underlying mechanisms. We found that *M. incognita* affected the leaf JA-related response and the metabolic profiles triggered by *S. exigua* herbivory, depending on the root infection cycle. Collectively, our study provides evidence that the impact of root infection by RKNs on AG herbivorous insects is dependent on the nematodes' infection cycle.

## MATERIALS AND METHODS

### Plant material and growing conditions

Tomato (*Solanum lycoperiscum* cv. 'Moneymaker') was used as a model plant in all experiments. We obtained tomato seeds from Intratuin B.V (Woerden, The Netherlands). Seeds were surface sterilized by immersion in 10 % sodium hypochlorite solution for four minutes. Subsequently, the seeds were rinsed four times with tap water. The sterilized seeds were placed on tap water moistened glass beads and allowed to germinate at 27 °C in the dark for three days, followed by four days in the light. When the seedlings were seven days old, they were transplanted into a 1:1 sand:soil mixture in 11x11x12 cm pots. Seedlings were grown in a glasshouse under 16 h light (26±3 °C) and 8 h dark (23±3 °C), according to Rodriguez-Saona *et al.* (2010). The plants were watered as required and supplemented with half-strength Hoagland solution (Hoagland and Arnon, 1938) weekly. The plants were grown in the glasshouse for three weeks before using them in experiments.

### Root and shoot herbivores

The RKN *M. incognita* was used as the root herbivore. Initial *M. incognita* eggs were kindly provided by Dr. Adriaan Verhage (Rijk Zwaan; De Lier, The Netherlands) and used to maintain a glasshouse stock colony on *S. lycopersicum*. The inoculum was started from a single egg mass, and when the infected plants were approximately 8 weeks old, eggs were harvested and used for experiments (Martínez-Medina et al., 2017). The generalist leaf chewer *S. exigua* was used as the shoot herbivore. *S.exigua* eggs were purchased from Entocare C.V. Biologische Gewasbescherming (Wageningen, The Netherlands). The colony was maintained in a growth chamber (CLF PlantClimatic, CLF PlantClimatics GmbH, Wertingen, Germany) and reared on an artificial diet at 25 °C, 45 % relative humidity with 12 h photoperiod cycle until use.

**Nematode infection and insect herbivore infestation**

Three weeks after transplanting, we infected the plants with the herbivores. The plants assigned for *M. incognita* infection were inoculated with approximately 3000 *M. incognita* eggs per plant suspended in tap water. The inoculation was performed by injecting 1 ml of an eggs' suspension (3000 egg $ml^{-1}$) into the soil close to the roots, according to Martínez-Medina *et al.* (2017). Plants that were not assigned for nematode inoculation were mock-inoculated with water. We set three study time points: 5, 15, and 30 days post nematode inoculation, coinciding with the following stages of nematodes' infection cycle; invasion (5 days), galling (15 days), and reproduction (30 days). At each time point, we infested the plants assigned for shoot herbivory with four first-instar *S. exigua* larvae (for the assessment of shoot herbivore performance); or one second-instar *S. exigua* larva (for molecular biology and chemical analyses).

**Bioassay for the assessment of *Spodoptera exigua* performance**

To assess the performance of *S. exigua* larvae when feeding on tomato plants challenged or not challenged with *M. incognita*, we conducted a bioassay including the study time points as described above. We used four first-instar *S. exigua* larvae. The *S. exigua* larvae were allowed to feed on plants challenged with *M. incognita* at the invasion, galling or reproduction stages, or on plants not infected with *M. incognita* until they reached the pupa stage. A total of 15 biological replicates were established per treatment. Larvae were first allowed to feed on the plant for six days without disturbance. After that, at two days interval, the larvae were removed, and their weight gain recorded. Larvae were returned to the same plant, on one leaf above the leaf where they were previously feeding on. This process was repeated all through until all surviving larvae either reached the pupa stage or died. The pupae were then collected and monitored until they hatched into moths. During the bioassay, we recorded data on larval weight, pupal weight, sex determination from the pupae, and duration of the pupation process until eclosion under 25 °C, 12 h photoperiod, and 45 % relative humidity regime. We also counted the number of roots galls formed by *M. incognita* at the galling and reproduction stages by visual inspection (Fig. S1).

**Bioassay for the assessment of tomato defensive and nutritional status**

To assess the impact of *M. incognita* root infection and *S. exigua* caterpillar feeding on tomato leaf defenses and elemental carbon (N) and nitrogen (N) content, we conducted a bioassay including the study time points as described above. We used a single second-instar *S. exigua* caterpillar. On each plant, the *S. exigua* caterpillar was contained on the leaf using a 2.5 cm wide clip cage (Bandoly and Steppuhn, 2016). In plants without leaf herbivory, an empty clip cage was set on a similar leaf as in plants with leaf herbivory. Caterpillars were allowed to feed on plants challenged with *M. incognita* at the invasion, galling or reproduction stages, or on not *M. incognita* infected plants for 24 h. Afterward, the damaged leaf was

harvested and stored at –80 °C for molecular biology and chemical analyses. For the analysis of trypsin protease inhibitor activity (TPI), we allowed *S. exigua* to feed on the plant for 48 h, according to Steppuhn and Baldwin (2007) and Bandoly *et al.* (2015).

**Determination of phytohormones concentrations**

We extracted plant hormones according to Machado *et al.* (2013) with slight modification, using ethyl acetate containing the internal standards: 40 ng $D_6$-JA and 40 ng $D_6$-JA-Ile as the solvent. The levels of 12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA) and jasmonyl-*L*-isoleucine (JA-Ile) were analyzed by using liquid chromatography (Bruker Advance UHPLC, Bremen, Germany) coupled to a mass spectrometer (Bruker Elite EvoQ Triple quadrupole, Bremen, Germany) (LC/MS EVOQ), as described by (Schäfer et al., 2016). The separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6x50 mm, 1.8 µm, 80 Å, Agilent technologies, Santa Clara, CA, USA), according to Machado *et al.* (2013). Data acquisition and processing were performed using the 'MS data Review' software (Bruker MS Workstation, version 8.2). Phytohormone levels were calculated based on the peak area of the corresponding internal standard and the amount of fresh weight of the leaf material ($ng^{-1}$ $mg^{-1}$ FW).

**Quantitative polymerase chain reaction analysis (qRT-PCR)**

Total RNA was extracted from ~100 mg (fresh weight) leaf material, according to Oñate-Sánchez and Vicente-Carbajosa (2008). First-strand cDNA was synthesized from 1 µg DNase free RNA using Revert Aid H-minus RT (Thermo Scientific) following the manufacturer's instructions. Real-time quantitative qPCR reactions and relative quantification of specific *m*RNA levels were performed according to Martínez-Medina *et al.* (2017) by using a CFX 384 Real-Time PCR system (Bio-Rad Laboratories Inc. USA) and the gene-specific primers described in Table S1. The data obtained were normalized using the housekeeping gene *SIEF X14449* which encodes for the tomato elongation factor 1α (Miranda *et al.,* 2013; Martínez-Medina *et al.,* 2017). The normalized data were further processed by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

**Trypsin protease inhibitor activity analysis**

To evaluate the trypsin protease inhibitor (TPI) activity in tomato leaves, we extracted total protein from 20 mg freeze-dried leaf material. The leaf samples were harvested 48 h after *S. exigua* herbivory, according to Bandoly *et al.* (2015). The extraction and quantification process was carried out according to the radial diffusion method described by van Dam *et al.* (2001) and Bandoly *et al.* (2015).

**Determination of elemental carbon and nitrogen concentrations**

Freeze-dried leaf material (~10 mg) was used for the determination of the elemental carbon and nitrogen concentration in percentages. The samples were weighed into tin bowls and carefully compressed into a circular pellet. The pellets were incinerated and detected by a Thermal Conductivity Detector (TCD) in an elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany), according to Moreno-Pedraza *et al.* (2019).

**Metabolites extraction and data processing**

To analyze the changes in tomato leaf metabolome, we extracted metabolites from ~20 mg (dry weight) leaf material. The extraction, quantification and data analysis of the metabolites was carried out as described by De Vos *et al.*, (2012); Rogachev and Aharoni (2012) and Moreno-Pedraza *et al.* (2019) with some modifications. We extracted twice every sample and combined the supernatants. We transferred 200 µl of the combined extracts into 2 ml HPLC vial and added 800 µl of the extraction buffer to obtain a 1:5 dilution for each sample. We further prepared 1:50 dilution of each sample by transferring 100 µl from each of the 1:5 dilutions into a new 2 ml HPLC vial and added 900 µl of the extraction buffer. The 1:50 dilution allowed us to correctly detect the tomatine peak without exceeding the mass analyzer detection limit. We separated and characterized compounds by injecting 1 µl of each extract from the two dilutions (1:5 and 1:50) in a UPLC (Dionex 3000, Thermo Scientific). The chromatograph was equipped with a C18 column (Acclaim TM RSLC 120), 2.1 x150 mm external dimension, 2.2 µm particle size, and 120 Å pore size. The column was kept at 40 °C. The mobile phases (LC-MS grade solvents) were composed of solvent A: 0.05 % (v/v) aqueous formic acid and solvent B: 0.05% (v/v) formic acid in acetonitrile. The multi-step gradient for solvent B was; 0−1 min 5 %, 1−4 min 28 %, 4−10 min 36 %, 10−12 min 95 %, 12−14 min 95 %, 14−16 min 5 %, 16−18 min 5 %. The flow was 400 µl $min^{-1}$. We detected compounds using a maXis impact HD MS-qToF (Bruker Daltonics). Data were acquired in positive mode with similar settings to Moreno-Pedraza *et al.* (2019). We processed the data with MS-Dial, according to Moreno-Pedraza *et al.* (2019) with slight modifications for feature detection, retention time correction, and feature alignment. The parameters were; mass accuracy: MS1 tolerance = 0.01 Da, retention time begin = 0.7 min, retention time-end = 10 min, mass range begin = 50 mass to charge ratio (*m*/*z*), mass range end = 1500 *m*/*z*; peak detection parameters: minimum peak height = 1000 amplitude, mass slice width = 0.1 Da, smoothing method=linear weighted moving average, smoothing level = 3 scans, minimum peak width = 5 scans; alignment parameters settings: retention time tolerance = 0.05 min, MS1 tolerance = 0.015 Da. We normalized the alignments against the total ion chromatogram. We exported the normalized data matrix containing all the alignments as a .txt file (spectra type = centroid). We predicted metabolites by interpreting mass spectral features and by comparison against mass spectra deposited in the Mass Bank of North America database.

**Statistical analysis**

Datasets were analyzed by using R software v 3.6.1 (R development Core Team 2019) unless indicated otherwise. For the performance datasets, we used one-way ANOVA for statistical computations and detected differences between groups using student *t*-test ($P \leq 0.05$) and Chi-square for the sex ratio dataset. In the case of defense responses datasets, we used two-way ANOVA linear models consisting of *M. incognita*, *S. exigua*, and their interaction as model explanatory factors. We detected differences between groups by Tukeys honest significant difference (HSD) for multiple comparisons ($P \leq 0.05$). All datasets were tested for normality and homogeneity of variance via visual inspection using Q-Q plots. In the case where ANOVA assumptions were violated, the data were transformed appropriately, as indicated in the legends of figures and tables.

## RESULTS

**Root infection by *Meloidogyne incognita* facilitates *Spodoptera exigua* performance during the nematode galling stage**

To analyze the influence of the nematodes' infection cycle on shoot herbivores, we carried out a performance bioassay in glasshouse involving *M. incognita* and *S. exigua* herbivory. We first challenged the tomato plants with *M. incognita*, and at different stages of infection, invasion, galling, and reproduction, we infested each shoot with four first-instar *S. exigua* larvae. We assessed the performance of *S. exigua* during continuous feeding. We found that in *M. incognita* infected plants at the invasion and reproduction stages, *S. exigua* larval and pupal weight gain was similar to that observed in control plants (Fig. 1A,B,G,H, Table S2). Moreover, neither the time of pupation nor the sex ratios of the emerging moths were significantly affected by *M. incognita* root infection at these stages (Fig. 1C,I, Table 1, Table S2). Altogether these observations indicate that *M. incognita* root infection at the invasion and reproduction stages did not affect the performance of *S. exigua*. By contrast, when *M. incognita* was in the galling stage, we observed a strong impact of *M. incognita* root infection on *S. exigua* performance. Our data showed an increase (although not statistically significant) in *S. exigua* larval weight on plants infected with *M. incognita* compared to control plants (Fig. 1D, Table S2). In addition, we observed significantly higher pupal weight (Fig. 1E, Table S2) and shorter pupation period (Fig. 1F, Table S2) in *S. exigua* pupae collected from *M. incognita* infected plants compared to those from controls. Moreover, we found a significantly higher proportion of emerging female *S. exigua* moths on *M. incognita* infected plants compared to controls (Table 1B). Overall, these results support our hypothesis that the impact of *M. incognita* root infection on AG feeding *S. exigua* is dependent on the nematodes' infection cycle. In our study, root infection by *M. incognita* facilitated the performance of *S. exigua,* specifically during the galling stage.

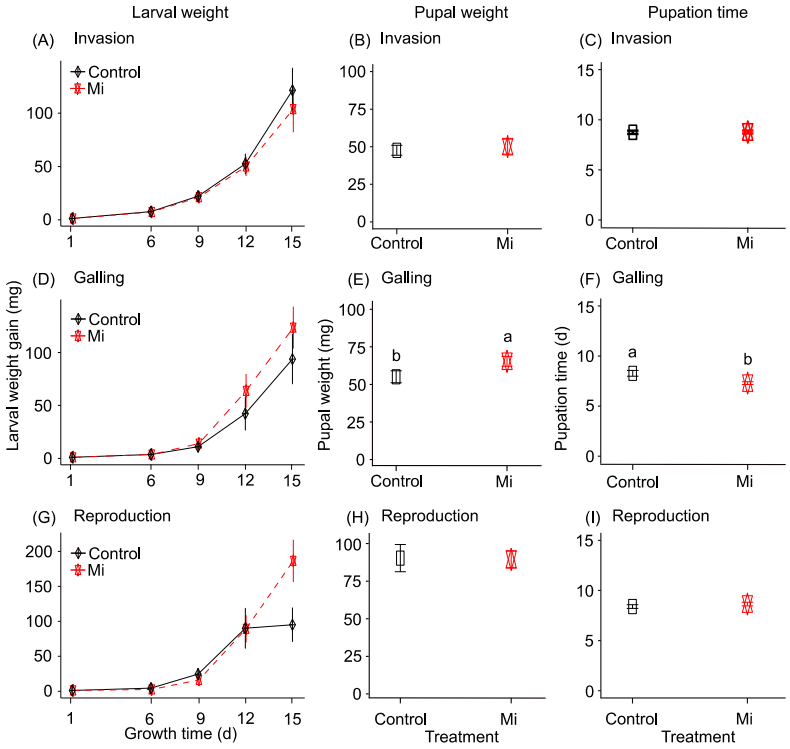


**Fig. 1 Impact of root infection by *Meloidogyne incognita* on the performance of *Spodoptera exigua*.** *Spodoptera exigua* larval weight gain (A,D,G), pupal weight (B,E,H) and pupation time (C,F,I) were measured in herbivores feeding on leaves of control plants, and from leaves of plants infected in roots with *Meloidogyne incognita* (Mi) at the invasion (A,B,C), galling (D,E,F) and reproduction (G,H,I) stages. Data are the mean ± standard error ($n$=15). Different letters indicate significant differences between treatments, inferred by student *t-test* at $p \leq 0.05$.

**Table 1. Chi-square test for the equality of *Spodoptera exigua* moths sex ratio.** Sex ratios were determined from *Spodoptera exigua* pupae collected from tomato plants without root infection (Control) and infected with *Meloidogyne incognita* (Mi). *Spodoptera exigua* infestation on Mi plants was performed either at the nematodes' invasion (A), galling (B), or reproduction (C) stages. Data are the numbers of sex ratios counted per treatment and were analyzed using one-way ANOVA. Asterisks indicate significant differences between the study treatments at each infection stage: Tukey HSD multiple comparison test at * $p \leq 0.05$; ** $p \leq 0.01$. Statistically significant effects are indicated in bold.

| Treatments | | Male | Female | Total per infection stage | Statistics | |
|---|---|---|---|---|---|---|
| | | | | | $\chi^2$ | $p \leq 0.05$ |
| (A) | Control | 6 | 3 | 18 | 3.7387 | 0.053 |
| | Mi | 2 | 7 | | | |
| (B) | Control | 7 | 5 | 30 | 4.0594 | **0.044** |
| | Mi** | 4 | 14 | | | |
| (C) | Control | 4 | 5 | 17 | 1.6721 | 0.196 |
| | Mi | 6 | 2 | | | |
| glm ANOVA results | | | | | $\chi^2$ | $p \leq 0.05$ |
| Time | | | | | 1.7364 | 0.420 |
| Mi | | | | | 2.8735 | 0.090 |
| Time*Mi | | | | | 6.5967 | **0.037** |

§; glm: generalized linear model, time: the nematode infection cycle stages (invasion, galling, reproduction), Mi: *Meloidogyne incognita*, $\chi^2$: chi-square.

**Root infection by *Meloidogyne incognita* modulates jasmonates-related shoot responses triggered by *Spodoptera exigua* feeding**

Jasmonates (JAs)-mediated defense responses are proposed to regulate the interaction between root-feeding nematodes and foliar feeding herbivores (Erb et al., 2012; Wasternack and Strnad, 2016). We investigated whether the impact of *M. incognita* on *S. exigua* performance is related to the modulation of JAs and related defenses marker genes at the different nematodes' root infection stages. Our data indicate that *M. incognita* root infection did not directly affect the concentration of OPDA, JA, and JA-Ile in tomato leaves compared to controls, regardless of the infection stages (Fig. 2, Table S3). In line with this, *M. incognita* infection did not directly affect the expression of the JAs marker genes *LoxD* (*Lipoxygenase D*), *PS* (*Prosystemin*), and *PI II* (*Proteinase inhibitor II*) (Fig. 3, Table S4) and the activity of trypsin protease inhibitor (TPI) (Fig. 4, Table S5) compared to control plants, regardless of the infection stage. These results suggest that *M. incognita* infection does not directly activate the JAs-related responses in tomato leaves. As expected, *S. exigua* leaf herbivory resulted in higher concentrations of OPDA, JA, and JA-Ile in tomato leaves compared to controls (Fig. 2, Table S3). In accordance, the expression of *LoxD*, *PS,* and *PI II* (Fig. 3, Table S4) and activity of TPI (Fig. 4, Table S5) was higher in plants challenged with *S. exigua* compared to control plants.

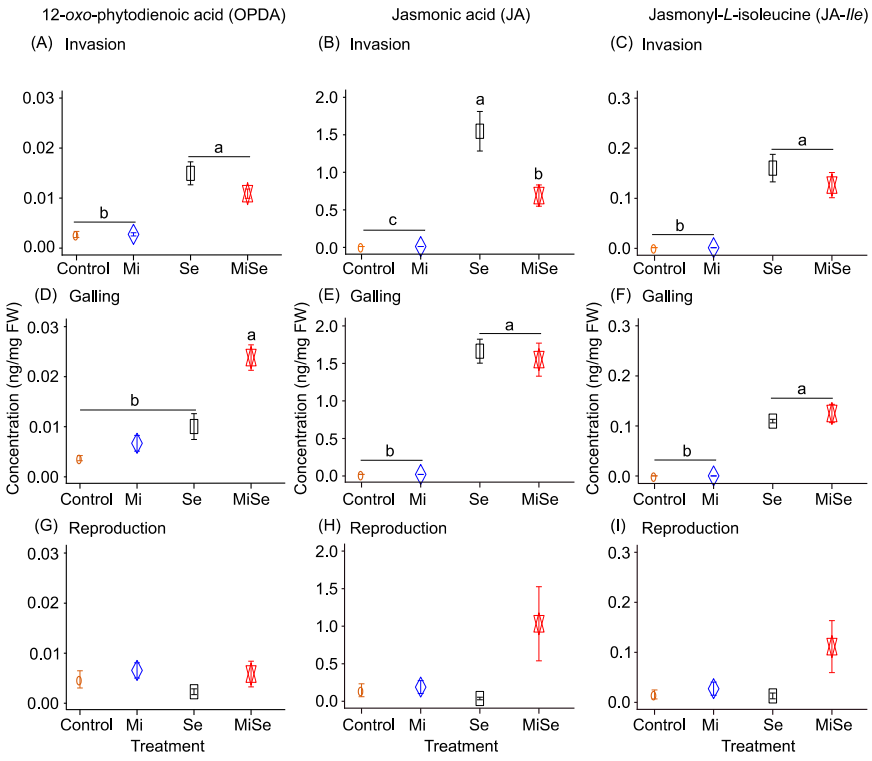


**Fig. 2 Concentrations of jasmonates in tomato leaves upon below and aboveground herbivory.** Concentrations of 12-*oxo*-phytodienoic acid (OPDA) (A,D,G), jasmonic acid (JA) (B,E,H), and jasmonyl-*L*-isoleucine (JA-Ile) (C,F,I) were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A,B,C), galling (D,E,F) or reproduction (G,H,I) stages. Samples were taken 24 hours after *S. exigua* feeding. Data are the mean ± standard error ($n$=8-10). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

In tomato plants challenged with both *M. incognita* and *S. exigua,* we found that at the nematodes' invasion stage, *M. incognita* root infection significantly reduced JA levels induced by *S. exigua* herbivory compared to plants challenged with *S. exigua* alone (Fig. 2B, Table S3). *Spodoptera exigua*-induced OPDA and JA-Ile levels were only slightly attenuated on plants infected by *M. incognita* (Fig. 2A,C, Table S3). Likewise, a significantly lower expression of *PI II* was found in double infected plants compared to that found in plants challenged with *S. exigua* alone (Fig. 3C, Table S4). By contrast, *LoxD* and *PS* expression increased in double infected plants compared to the expression in plants challenged with *S. exigua* alone (Fig. 3A,B, Table S4). Despite the increase in gene expression, we did not find differences in the activity of TPI between double infected plants, and those challenged with *S. exigua* alone (Fig. 4A, Table S5). At the galling stage,

we found an increase in OPDA levels in double infected plants compared to plants challenged with *S. exigua* alone (Fig. 2D, Table S3). In correspondence, significant upregulation in the expression of JAs biosynthesis marker gene *LoxD* was observed in double infected plants, compared to plants challenged with *S. exigua* alone (Fig. 3D Table S4). The *S. exigua* induced JA and JA-Ile remained similar in double infected plants and those challenged with *S. exigua* alone (Fig. 2E,F, Table S3). In agreement, *M. incognita* root infection did not affect the expression of *PI II* and *PS* triggered by *S. exigua* herbivory (Fig. 3E,F, Table S4). The activity of TPI in double infected plants did not differ compared to plants challenged with *S. exigua* alone (Fig. 4B, Table S5). At the reproduction stage, double infection by *M. incognita* and *S. exigua* did not affect the levels of JAs compared to plants challenged with *S. exigua* alone (Fig. 2G-I, Table S3). In accordance, the expression of *LoxD*, *PS, and PI II* in double infected plants remained similar to those in plants challenged with *S. exigua* alone (Fig. 3G-I Table S4). Notably, the activity of TPI in double infected plants was significantly reduced compared to observation in plants challenged with *S. exigua* alone (Fig. 4C, Table S5). Collectively, the phytohormone, transcript, and TPI activity data indicate that the *M. incognita* root infection cycle can modulate, at least partially, the JAs- responses elicited in leaves by *S. exigua* herbivory.

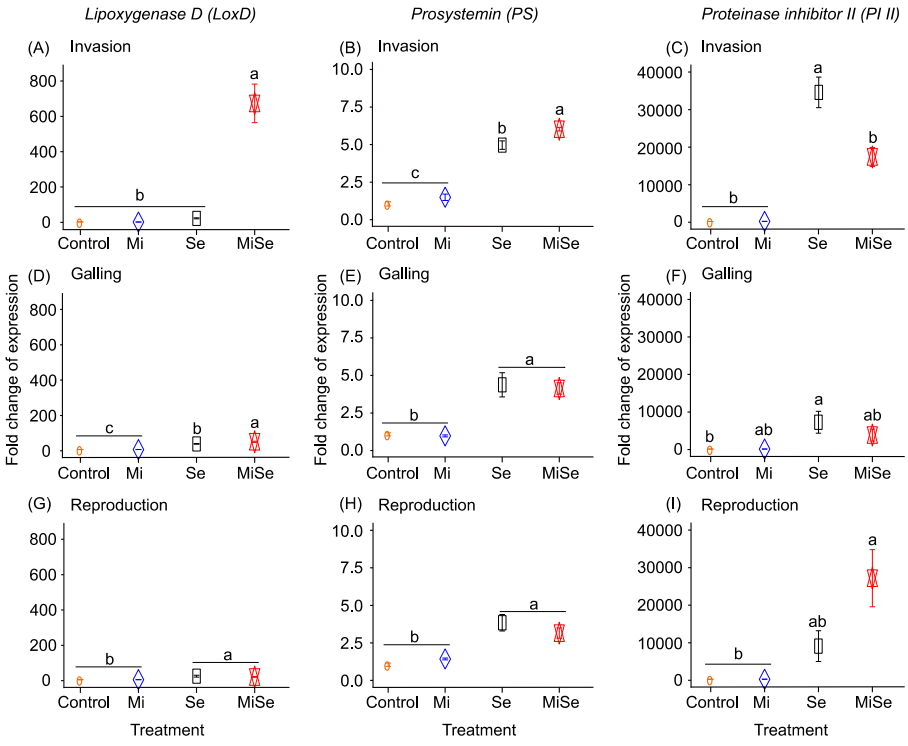

**Fig. 3 Expression patterns of jasmonate-dependent defense marker genes in tomato plants upon below and aboveground herbivory.** Relative expression of *Lipoxygenase D* (*LoxD*) (A,D,G), *Prosystemin* (*PS*) (B,E,H), and *Proteinase inhibitor II* (*PI II*) (C,F,I) were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A,B,C), galling (D,E,F) or reproduction (G,H,I) stages. Samples were taken 24 hours after *S. exigua* feeding. Data are the mean ± standard error (*n*=8-10). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

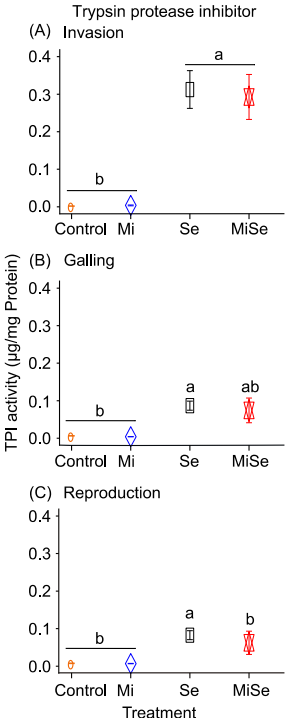


**Fig. 4 Trypsin protease inhibition activity in tomato leaves upon below and aboveground herbivory.** Trypsin protease inhibition activity (A,B,C) was measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A), galling (B), or reproduction (C) stages. Samples were taken 48 hours after *S. exigua* feeding. Data are the mean ± standard error (*n*=5). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

**Root infection by *Meloidogyne incognita* systemically alters elemental carbon and nitrogen ratios in tomato leaves during the nematodes' galling stage**

The ratio between the amount of carbon (C) and nitrogen (N) is an essential indicator of plant quality for insect herbivores (Crafts-Brandner, 2002). We tested whether root infection by *M. incognita* affects the concentrations of elemental C and N in tomato leaves. As shown in Tables 2 and S6, *M. incognita* root infection did not directly affect C and N in the leaves compared to controls, regardless of the infection stage. Similarly, *S. exigua* herbivory did not affect the leaf C and N compared to control plants. The shoot C and N concentration in plants challenged with *M. incognita* and *S. exigua* remained similar to that observed in plants challenged with *S. exigua* alone.

In the case of the C/N ratio, we did not observe significant differences between plants infected with *M. incognita* at invasion or reproduction stages compared to control plants (Tables 2,S6). Notably, our data showed that *M. incognita* root infection increased the C/N ratio in tomato leaves, at the galling stage compared to control plants (Tables 2, S6). *Spodoptera exigua* herbivory did not affect C/N ratio compared to control plants (Tables 2, S6). The C/N ratio in plants challenged with both *M. incognita* and *S. exigua* remained similar to that observed in plants challenged with *S. exigua* alone throughout the nematodes' infection cycle (Tables 2, S6). These results show that root infection by *M. incognita* affects the shoot C/N ratio in dependence with the nematodes' infection cycle.

**Table 2. Concentrations of elemental carbon and nitrogen (in percentages) and carbon/nitrogen ratio in tomato leaves upon below and aboveground herbivory.** Concentrations of elemental carbon (C), and nitrogen (N), and C/N ratio were determined in leaves of tomato plants without herbivores (control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after *S. exigua* feeding. Data are the mean ± standard error ($n$=3). Statistically significant means are indicated in bold.

| Parameter | Treatment | Invasion | Galling | Reproduction |
|---|---|---|---|---|
| C | Control | 40.80±0.30 | 42.65±0.75 | 43.13±0.69 |
| | Mi | 41.29±0.25 | 42.87±0.60 | 43.10±0.38 |
| | Se | 40.35±0.22 | 42.62±0.47 | 42.67±0.43 |
| | MiSe | 41.03±0.35 | 42.89±0.33 | 43.10±0.43 |
| N | Control | 4.74±0.25 | 3.62±0.17 | 2.62±0.30 |
| | Mi | 4.57±0.19 | 3.06±0.34 | 2.42±0.23 |
| | Se | 4.60±0.33 | 3.44±0.21 | 2.63±0.28 |
| | MiSe | 4.67±0.34 | 3.14±0.27 | 2.28±0.18 |
| C/N ratio | Control | 8.83±0.51 | 12.0±0.64 | 18.31±2.07 |
| | Mi | 9.17±0.41 | **15.37±1.55** | 19.57±2.39 |
| | Se | 9.21±0.76 | 12.79±0.82 | 17.74±1.95 |
| | MiSe | 9.21±0.74 | 14.51±1.28 | 20.04±1.90 |

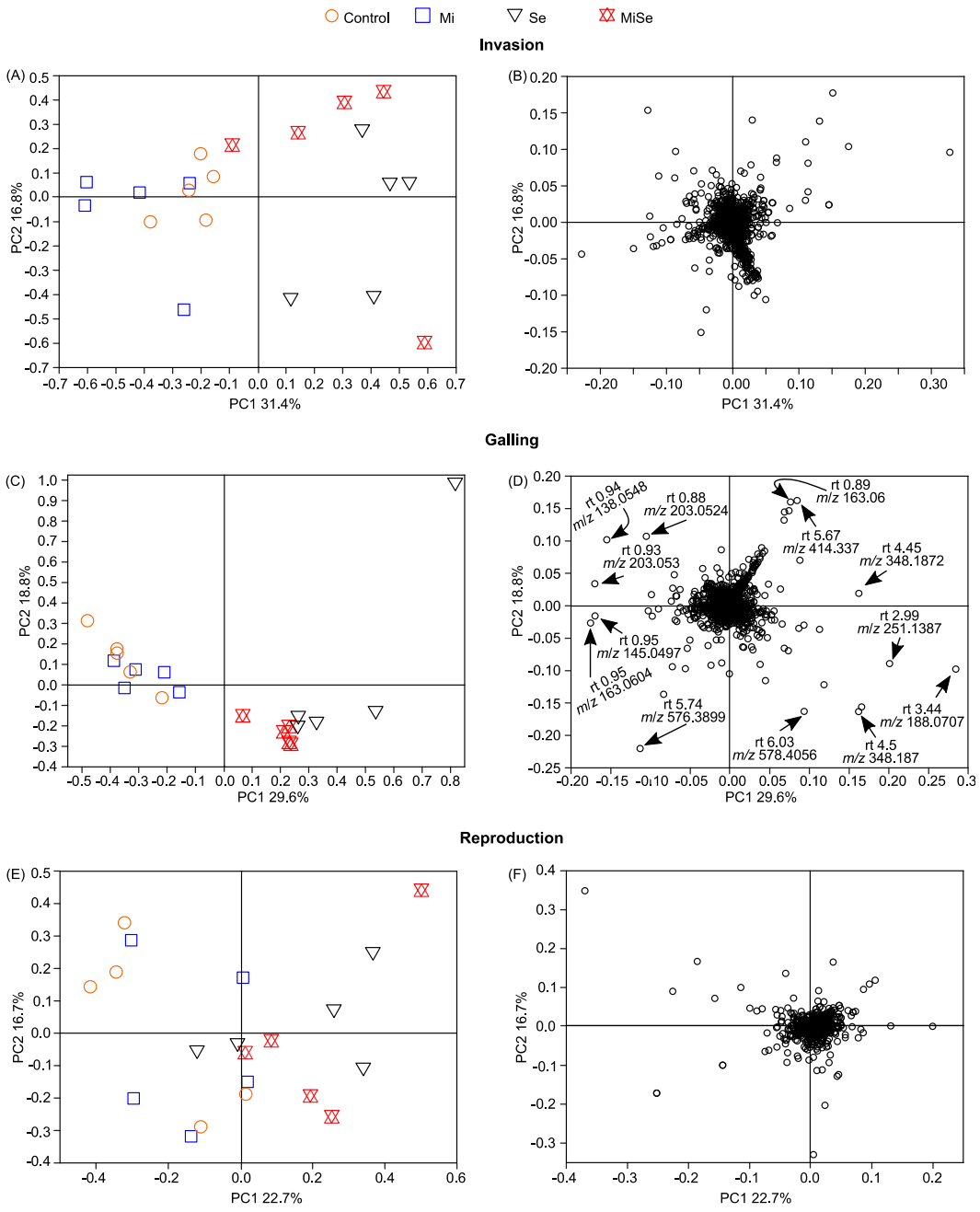


**Fig. 5 Principal component analysis; scores and loadings plots of leaf metabolic profiles in tomato plants upon below and aboveground herbivory.** Metabolic profiles analyzed in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A,B), galling (C,D), or reproduction (E,F) stages. Samples were taken 24 hours after *S. exigua* feeding. Panels (A,C,E) show scores plots of principal component (PC) 1 and 2 showing the separation between treatments. Panels (B,D,F) show the loading plots displaying the projection of each LC-MS feature. Arrows in panel D point to the most variable loadings selected for structural prediction.

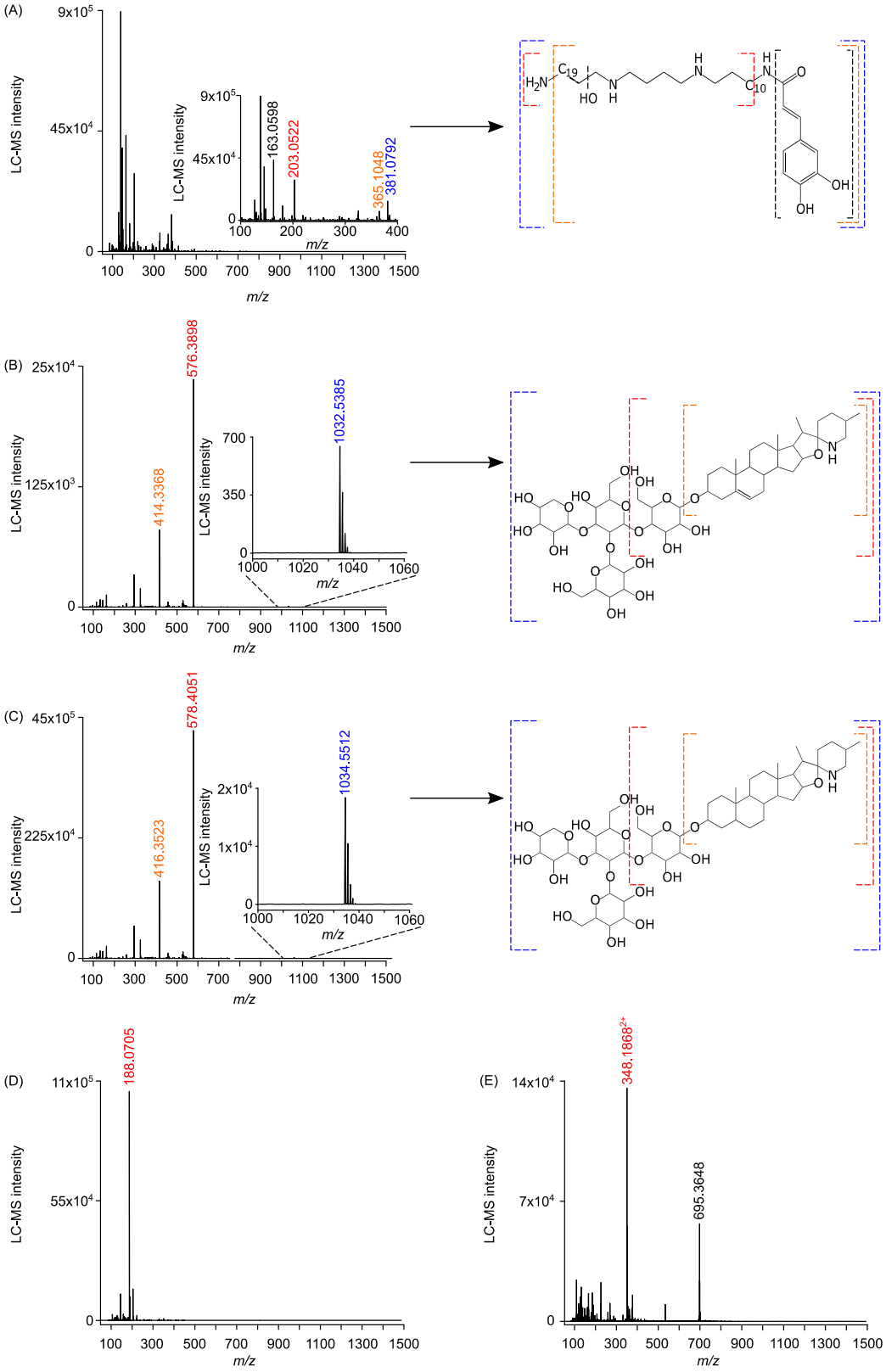
(A)
LC-MS intensity
m/z
163.0598
203.0522
365.1048
381.0792
(B)
576.3898
414.3368
1032.5385
(C)
578.4051
416.3523
1034.5512
(D)
188.0705
(E)
348.1868
695.3648

**Fig. 6 Mass spectra and structures of the predicted metabolites**. Mass spectra and predicted structures of four highly variable metabolites selected at the nematodes' galling stage. Panels show the LC-MS intensities per metabolite detected by LC-MS in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected plants with both herbivores (MiSe). Panels (A), (B), and (C) represent polyamine conjugated to a phenylpropanoid, steroidal glycoalkaloids *α*-dehydrotomatine and *α*-tomatine, respectively. Panels (D) and (E) represent two unknown metabolites that were among the most variable loadings. In panels A, B and C, the numbers in blue represent the *m*/*z* of the predicted parent ion $[M+H]^{1+}$, in red represent the *m*/*z* of fragments that would have originated from the parent ion and are reported in the study, and in orange and black represent *m*/z of other fragments likewise would have originated from the parent ion. In panel D the number in red represents the *m*/*z* value reported in the study. In panel E the number in black represents a *m*/*z* value, and the number in red its corresponding to *m*/2*z* (if *m*/*z* 695.3648 represents the molecular ion then *m*/*z* 348.1868 represents the species $[M+2H]^{2+}$).

**Root infection by *Meloidogyne incognita* alters the shoot metabolic profiles triggered by *Spodoptera exigua* feeding**

We studied whether root infection by *M. incognita* affected the metabolic profile triggered by *S. exigua* herbivory in leaves. With this aim, we analyzed the impact of *M. incognita* root infection at the invasion, galling, and reproduction stages on the metabolic profile triggered by *S. exigua* herbivory (Fig. 5). At the invasion stage, the first PC explained 31.4 % of the total variance and revealed two clusters: control and *M. incognita* infected plants in one group, and *S. exigua* and double infected plants on the other group (Fig. 5A). At the galling stage, the first PC explained 29.6 % of the total variance and revealed a separation of plants into two clusters: control and *M. incognita* infected plants were all projected to the left while all plants treated with *S. exigua* were to the right of the plot (Fig. 5C). In addition, we observed a separation between the double infected plants from plant challenged with *S. exigua* alone. At the reproduction stage, the first two components explained 39.4 % of the total variance, but we did not observe a clear separation between the groups (Fig. 5E). These results show that the impact of *S. exigua* feeding on tomato leaves metabolomes is stronger than the effect of *M. incognita* infection, at least during the invasion and galling stages. Our results further indicate that root infection by *M. incognita* partially alters the metabolic profiles triggered by *S. exigua* feeding, specifically during the galling stage.

**Root infection by *Meloidogyne incognita* at the galling stage alters the concentration of a polyamine conjugate and an unknown metabolite triggered by *Spodoptera exigua* feeding**

The effect of *M. incognita* root infection on *S. exigua* performance and the leaf metabolome was strongest at the nematodes' galling stage (Fig. 1,5C). For this reason, we analyzed in more detail the metabolic profiles at the galling stage. On the loadings plot Fig. 5D we selected the molecular features that were projected farthest from the center of the plot as they exhibit the highest variability and underlie

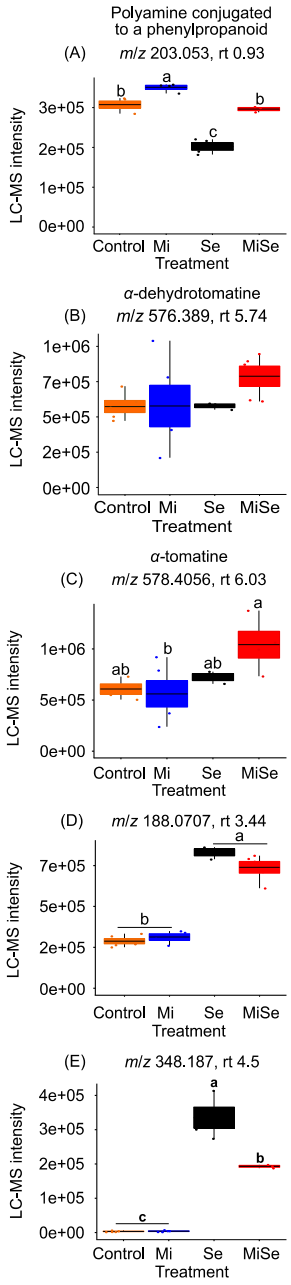


**Fig. 7 LC-MS intensities of the selected metabolites in tomato leaves upon below and aboveground herbivory.** LC-MS intensities and mass to charge ratio (*m*/*z*) and retention time (rt) in minutes of polyamine conjugated to a phenylpropanoid (A), α-dehydrotomatine (B), α-tomatine (C) and unknown metabolites (D,E) measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' galling stage. Samples were taken 24 hours after *S. exigua* feeding. Data are the mean ± standard error ($n$=5). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

the separation between the treatments found in Fig. 5C. Using the *m*/*z* value for each selected feature, we checked for signals in the chromatograms and picked out only features with a conspicuous LC-MS peak and interpreted the mass spectra. We predicted structures of a polyamine conjugated to a phenylpropanoid with *m*/*z* 203.053 at 0.93 min rt (Fig. 5D,6A), and two steroidal glycoalkaloids; α-dehydrotomatine with *m*/*z* 576.389 at 5.74 min rt, and α-tomatine with *m*/*z* 578.4056 at 6.03 min rt (Fig. 5D,6B). Two other selected features with *m*/*z* 188.0707 at 3.44 min rt, and *m*/*z* 348.187 at 4.5 min rt, had a conspicuous LC-MS peak, but we were unable to predict their structures (Fig. 5D,6C,D).

Next, we plotted the LC-MS intensities for the corresponding *m*/*z* values for both the predicted and unknown metabolites (Fig. 7). We found that *M. incognita* infection directly increased the concentration of the polyamine conjugate (Fig. 7A, Table S7), but it had no direct effect on the concentration of the steroidal glycoalkaloids; α-dehydrotomatine and α-tomatine, and the two unknown metabolites compared to controls (Fig. 7B,C,D,E, Table S7). *S. exigua* herbivory triggered a decrease in the concentration of the polyamine conjugate (Fig. 7A, Table S7), and did not affect the concentration of the steroidal glycoalkaloids; α-dehydrotomatine and α-tomatine compared to controls (Fig. 7B,C, Table S7). However, *S. exigua* herbivory increased the concentration of the two unknown metabolites compared to controls (Fig. 7D,E, Table S7).

In plants challenged with both *M. incognita* and *S. exigua*, the concentration of the polyamine conjugate increased compared to plants challenged with *S. exigua* alone (Fig. 7A, Table S7). Double

infection did not affect the concentrations of the steroidal glycoalkaloids; α-dehydrotomatine and α-tomatine, and the unknown metabolite with *m*/*z* 188.0707 at 3.44 min rt compared to plants challenged with *S. exigua* alone (Fig. 7B,C,D, Table S7). We observed that the concentration of the unknown metabolites with *m*/*z* 348.187 at 4.5 min rt was significantly decreased in double infected plants compared to plants challenged with *S. exigua* alone (Fig. 7E, Table S7).

## DISCUSSION

Here, we demonstrated that the impact of the RKN *M. incognita* on the performance of the AG herbivore *S. exigua* is strongly influenced by the nematodes' infection cycle. Our experimental design allowed us to identify that, specifically at the galling stage, root infection by *M. incognita* facilitated the performance of the shoot herbivore. By contrast, *M. incognita* root infection did not affect the growth and the performance of *S. exigua* when the nematode was in either the invasion or reproduction stage. Previous studies have demonstrated the influence of RKNs on the performance of herbivores feeding on shoots (Carter-Wientjes et al., 2004; Kaplan et al., 2008b, 2009; Tiwari et al., 2009; Arce et al., 2017). Notably, these studies show a variety of interaction outcomes for the AG herbivores. For example, Kaplan *et al.* (2008) demonstrated that infection by *M. incognita* in tobacco roots increased the larval weight of the AG herbivores *Trichoplusia ni* and *S. exigua*, while it did not affect the performance of *M. sexta*. On the other hand, *M. incognita* root infection of soybean resulted in inconsistent effects on the performance of the AG herbivore *Pseudoplusia includens* (Carter-Wientjes et al., 2004). These studies propose that the susceptibility of the host plant to the nematode infection and the identity of the herbivores are significant factors driving variation in the interaction outcomes for the AG herbivores (Wurst and van der Putten, 2007; Sarmento et al., 2011; Kyndt et al., 2012a; Wondafrash et al., 2013; Biere and Goverse, 2016). Our findings point to the RKN infection cycle as a further key factor influencing the outcome of the interaction between RKN and AG herbivores when sharing a host plant. This is not surprising as the plant interaction with RKNs is highly dynamic, and root responses to RKNs profoundly differ between the initial and advanced stages of the infection (Kammerhofer *et al.*, 2015*b*; Martínez-Medina *et al.*, 2017). Differences in root responses may lead to different systemic responses, and thereby have different effects on insect herbivores feeding on AG plant tissues. The enhanced *S. exigua* performance on *M. incognita* root-infected plants at the galling stage was not accompanied by a higher leaf consumption; the presence of *M. incognita* did not affect *S. exigua* leaf consumption (Fig. S2). This indicates that the facilitation by *M. incognita* at the galling stage may have been mediated by an increase in leaf nutritional quality or by a suppression of the plant's ability to mount an effective defense against *S. exigua*.

Among the defense-related signaling pathways, the JA signaling pathway is proposed to play a major role in orchestrating AG-BG plant-mediated interactions between herbivores (Erb et al., 2008; Biere

and Goverse, 2016; van Dam et al., 2018; Karssemeijer et al., 2020). Indeed, the JA signaling pathway is considered to be one of the central pathways governing plant systemic responses to AG chewing herbivores and also root responses to RKN infection BG (Farmer and Ryan, 1992; Bhattarai et al., 2008; Hamamouch et al., 2011; Bosch et al., 2014b, 2014a; Fan et al., 2014; Gheysen and Mitchum, 2019). We investigated whether root infection by *M. incognita* altered the JAs triggered by *S. exigua* herbivory and whether this effect was modulated by the nematodes' infection cycle. We found that root infection by *M. incognita* alone did not directly affect the concentration of OPDA, JA, or JA-Ile, expression of JA signaling marker genes, or activity of TPI in leaves, regardless of the nematodes' infection cycle stages. This indicates that *M. incognita* root infection does not affect the JA signaling pathway systemically in the leaves of tomato plants. In contrast to our observation, both direct elicitation (Kafle et al., 2017; Wang et al., 2019) and repression (Hamamouch et al., 2011; Kyndt et al., 2012b) of the JA-pathway has been observed in leaves upon RKN root infection. Although we do not have a specific explanation for this variability of results, it highlights the complexity of the root-RKN interaction (Bird, 1974; Cabrera et al., 2015; Gheysen and Mitchum, 2019).

Whereas *M. incognita* root infection did not directly affect JAs in leaves, it affected the JAs-related responses triggered in leaves by *S. exigua* herbivory. The modulation of the *S. exigua*-induced JAs by *M. incognita* varied over the nematodes' infection cycle. For instance, *M. incognita* root infection at the invasion stage impaired the accumulation of JA and the transcriptional activation of *PI II* triggered by *S. exigua* feeding, suggesting the ability of *M. incognita* to repress the JA-related response triggered by *S. exigua*. By contrast, at the galling stage, *M. incognita* enhanced the accumulation of OPDA and the expression of the JA biosynthesis gene *LoxD* elicited by *S. exigua*, pointing to a priming effect by *M. incognita* infection on the JA-biosynthesis pathway (Martínez-Medina *et al.*, 2016). Moreover, *M. incognita* root infection at the reproduction stage enhanced the expression of *PI II* triggered by *S. exigua*, while it reduced the activity of TIP elicited by *S. exigua*. Collectively these results reinforce our hypothesis that the impact of *M. incognita* infection on AG defense responses triggered by shoot herbivores occurs in strict dependence on the nematodes' infection cycle.

Remarkably, the systemic modulation of the leaf JAs by *M. incognita* did not correlate with the performance of *S. exigua*. Indeed, we found a facilitation effect to *S. exigua* by *M. incognita* infection at the galling stage, which concurred with an enhancement of the accumulation of the JA precursor OPDA and the JA biosynthesis marker gene *LoxD*. OPDA has been shown to contribute to plant resistance against herbivory, independently of the JA/JA-Ile biosynthesis and signaling (Bosch et al., 2014a, 2014b). However, consistent with our observations, it was demonstrated that OPDA-mediated induction of resistance is not sufficient for conferring plant resistance against *S. exigua* herbivory (Bosch et al., 2014b). On the other hand, the impairment of *S. exigua*-triggered JA accumulation and *PI II* expression elicited by *M. incognita* at the invasion stage was not accompanied by any effect on *S. exigua* performance. This may

suggest that JA-triggered *PI II* does not have a major role in the performance of *S. exigua*. Along the same lines, Jongsma *et al.* (1995) found that *S. exigua* growth was unaffected by high levels of *PI II* in tobacco leaves. Altogether, these findings suggest the existence of additional mechanisms underlying the impact of root infection by *M. incognita* on the performance of *S. exigua*.

Metabolomics approaches provide an opportunity to assess local and systemic herbivore-induced changes in plant metabolic patterns without any prior assumption (Viant, 2008; Peters et al., 2018). We applied untargeted metabolomics to assess whether *M. incognita* root infection altered the shoot metabolome elicited by *S. exigua* herbivory and whether this effect was modulated by the nematodes' infection cycle. We found a stronger impact of *S. exigua* herbivory on tomato leaf metabolome compared to the impact of *M. incognita* root infection. Indeed, a strong overlap in the leaf metabolic profiles was found between control plants and *M. incognita*-infected plants, regardless of the infection cycle stages. These findings are in line with our observations on the minor direct impact of *M. incognita* on JAs described above, and reinforce our findings that the direct impact of *M. incognita* root infection on shoot chemistry is rather moderate. Moreover, the AG metabolic profiles triggered by *S. exigua* leaf herbivory were markedly different from those triggered by *M. incognita* root infection, especially at the invasion and galling stages. Although the identity of the metabolites altered in both interactions remains unknown, such differences may underlie the different feeding styles and life strategies of both herbivores (Wondafrash et al., 2013).

While the direct impact of root infection by *M. incognita* on shoot metabolic profiles was moderate, *M. incognita* altered at least partially the metabolic profiles triggered by *S. exigua* herbivory, at the nematodes' invasion and galling stages. Our results demonstrated that *M. incognita* at the galling stage facilitated *S. exigua* performance. Therefore, we focused specifically on the galling stage, to try to hypothesize relevant metabolites that might underlie this phenotype. Among the LC-MS features with the highest variability in the PCA, we predicted a polyamine conjugated to a phenylpropanoid. Although further analysis would be required, according to its mass spectrum, we speculate that it might be a derivative of spermine. Polyamine conjugates have been shown to have a prominent role in plant defense against herbivores. Accumulation of putrescine/spermidine polyamine conjugates was strongly induced in tobacco plants by herbivory, and this is coordinated by the transcription factor MYB8 (Kaur et al., 2010; Onkokesung et al., 2012). Moreover, *M. sexta* and *S. littoralis* feeding on systemically pre-induced leaves performed significantly better on ir-MYB8 plants lacking phenylpropanoid-polyamine conjugates compared with wild-type plants expressing high levels of phenylpropanoid-polyamine conjugates (Kaur et al., 2010). Remarkably, we found a significant decrease in the concentration of the predicted polyamine conjugate in leaves after *S. exigua* feeding. This decrease might be related to the ability of *S. exigua* to downregulate plant immune responses (Bandoly et al., 2015). In contrast, *M. incognita* root infection

stimulated the accumulation of this polyamine conjugate in leaves. It has been demonstrated that plant-parasitic nematodes can manipulate the biosynthesis of polyamines to promote infestation (Hewezi et al., 2010). Remarkably, *M. incognita* root infection counteracted the decrease in the concentration of the detected polyamine conjugate triggered by *S. exigua* feeding. Taking into consideration that we found a facilitation effect of *M. incognita* on the performance of *S. exigua*, we hypothesize that the predicted polyamine conjugate does not play a major role in plant defenses against *S. exigua*. The polyamine biosynthetic pathway is highly interconnected and plastic, leading to the biosynthesis of a broad spectrum of polyamine conjugates depending on the specific stress (Kaur et al., 2010; Onkokesung et al., 2012). It was further suggested that a mixture of various polyamine conjugates may be required to extert the maximal efficiency of polyamine conjugates against herbivores (Onkokesung et al., 2012). Whereas further studies are required to shed more information on the role of polyamines and their conjugates in AG-BG interactions, we hypothesize that this specific polyamine conjugate does not play a major role in the facilitation effect triggered by *M. incognita* on *S. exigua* performance

Besides the polyamine conjugate, we also predicted the steroidal glycoalkaloids *α*-dehydrotomatine and *α*-tomatine. We found that *S. exigua* feeding did not affect the accumulation of these steroidal glycoalkaloids in tomato leaves. It is noticeable that the accumulation of these steroidal glycoalkaloids was higher (although not statistically significant) in leaves of double infected plants compared to those challenged with *S. exigua* alone. Steroidal glycoalkaloids in *Solanum* species function as first-line defense metabolites against pathogens and herbivores (Güntner et al., 1997; Friedman, 2002; Ökmen et al., 2013; Carere et al., 2016; Chowański et al., 2016; Dahlin et al., 2017; Garcia et al., 2018). However, despite the observed increase in steroidal glycoalkaloids concentration in the double infected plants, *S. exigua* performed better in these plants. Secondary metabolites can vary in their effects on insect herbivores. For example, in potato accumulation of steroidal glycoalkaloids; *α*-solanine and *α*-chaconine reduces *S. exigua* growth (Kumar et al., 2016), while in black nightshade did not affect the phytophagous lady beetle *Henosepilachna vigintioctomaculata* (Hori et al., 2011). Interestingly, a previous study demonstrated that α-tomatine had little or no effect on food consumption, assimilation, or dietary utilization of the food by *S. exigua* larvae and other herbivores (Bloem et al., 1989). These studies demonstrate that steroidal glycoalkaloids can vary in their effects on insect herbivores. In our case, the results point out that the stronger accumulation of the steroidal glycoalkaloids in double infected plants did not affect the performance of *S. exigua*.

Among the most variable molecular features were also two metabolites with *m*/*z* 188.0707 at 3.44 min rt and *m*/*z* 348.187 at 4.5 min rt. A strong increase in the concentrations of these metabolites was observed after leaf herbivory by *S. exigua*. Although we were unable to predict the structures of these metabolites, we hypothesize that they might act as anti-herbivory defense compounds. It is remarkable that

in double infected plants, we observed a lower accumulation of both metabolites compared to plants challenged with *S. exigua* alone. Although additional analyses would be required, we hypothesize that this effect might underlie, at least partially, the facilitation effect observed at the nematode's galling stage.

Besides the changes in plant defense traits, the performance and population dynamics of AG insect herbivores also depend on the nutritive quality of the host plant (Awmack and Leather, 2002). It has been established that after herbivory, plants allocate C and N to specific tissues to be utilized for compensatory growth or defense of valuable plant parts (Creelman and Mullet, 1997; Wang et al., 2016; Kafle et al., 2017). Our results showed that *M. incognita* did not affect elemental C and N content significantly in leaves, but it did increase the C/N ratio, specifically at the nematodes' galling stage. Moreover, at the nematodes' galling stage, we observed a higher (although not statistically significant) C/N ratio in double infected plants compared to plants challenged with *S. exigua* alone. It is established that higher C/N ratios in plant tissues, generally reduces host plant quality for herbivores (Bryant et al., 1983; Luo et al., 2006; Dáder et al., 2016). However, we found an enhanced performance of *S. exigua* when feeding on plants infected by the nematodes' at the galling stage. We therefore speculate that this potential reduction in host plant quality mediated by *M. incognita* did not contribute to the facilitation effect observed.

In conclusion, our findings demonstrate that the impact of root infection by the RKN *M. incognita* on systemic defense responses, and the performance of the AG herbivore *S. exigua* is modulated by the nematodes' infection cycle. Based on these conclusions, we propose that it is crucial to consider the infection cycle of plant-parasitic nematodes in future studies dealing with AG-BG plant-mediated interactions.

## DATA STATEMENT

The datasets underlying this study will be open access. We have deposited the performance and defense response datasets in the iDiv data repository. These are; (1). "Mbaluto C., van Dam NM., and Martínez-Medina A. (2020). Impact of root-knot nematode (*Meloidogyne incognita*) root infection on the performance of *Spodoptera exigua*. iDiv Data Repository; https://idata.idiv.de/ddm/Data/ShowData/1839". (2). "Mbaluto C., van Dam NM., and Martínez-Medina A. (2020). Root infection by the root-knot nematode *(Meloidogyne incognita)* modulate aboveground defense responses triggered by *Spodoptera exigua*. iDiv Data Repository; https://idata.idiv.de/ddm/Data/ShowData/1833". The DOIs are currently under embargo until a decision on the manuscript has been made

## ACKNOWLEDGMENTS

All authors gratefully acknowledge the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118). CMM further acknowledges the Deutscher Akademischer Austauschdienst (DAAD) for a doctoral research grant (research grant No: 91607343). We thank Dr. Adriaan Verhage of Rijk-Zwaan, Netherlands, for providing the root-knot nematode eggs. We thank Dr. Katharina Grosser and Anne Maedicke for their technical support in molecular biology and chemical analyses. Dr. Ainhoa Martínez-Medina further acknowledges funding from the program for attracting talent to Salamanca from the Fundación Salamanca Ciudad de Cultura y Saberes and Ayuntamiento de Salamanca; and the program to support junior researchers to obtain third-party funding from Friedrich-Schiller-Universität Jena (DRM/2015-02).

## AUTHOR CONTRIBUTIONS

**CMM**; contributed to the conception of the idea, experimental design and conducting the experiments, processing of samples, data analysis, literature search and drafting the initial manuscript; **FV**; contributed through metabolomics data analysis and interpretation, and writing of the manuscript chemistry section; **NMvD and AMM**; contributed to the conception of the idea, experimental design, critical revisions of draft manuscript and approval of final manuscript for submission.

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## SUPPORTING INFORMATION

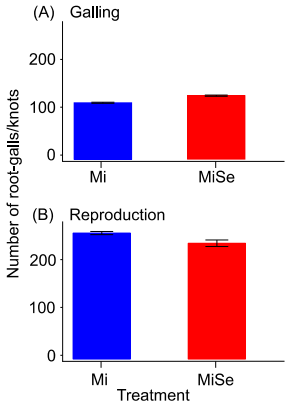


**Fig. S1 Number of *Meloidogyne incognita* galls in tomato roots**. The average number of root galls counted in tomato roots infected with *Meloidogyne incognita* alone (Mi) or double infected with *M. incognita* and *Spodoptera exigua* (MiSe). Roots were harvested at the nematodes' galling (A) and reproduction (B) stages. Data are the mean ± standard error ($n$=10).

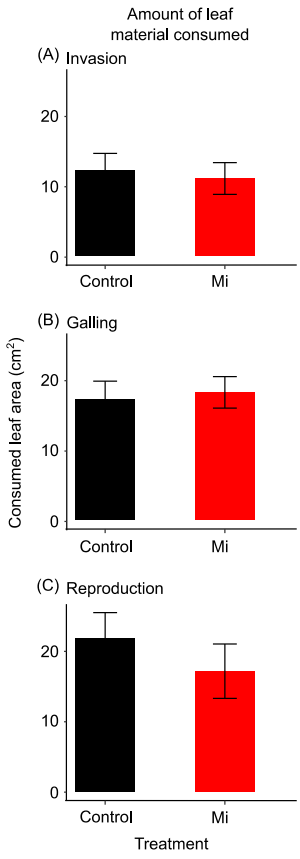


**Fig. S2 Amount of leaf material consumed by *Spodoptera exigua*.** The average feeding rate of *Spodoptera exigua* determined as leaf area in $cm^2$. Second-instar *S. exigua* larvae were added and allowed to feed for five days on tomato plants without root infection (Control) or infected with *Meloidogyne incognita* (Mi). The leaves were harvested at the nematodes' invasion (A), galling (B), and reproduction (C) stages for the determination of leaf areas. Data are the mean ± standard error ($n$=10).

**Table S1 List of primer sequences used for qRT PCR reactions**

| Target gene | Primer sequences (5'→3') |
|---|---|
| *Lipoxygenase D* (*LoxD*)[a] | Fw: GGCTTTATTTCACACAGAGATA<br>Rev: ATGTGCTGCCAATATAAATGGTTCC |
| *Prosystemin (PS*)[b] | Fw: AATTTGTCTCCCGTTAGA<br>Rev: AGCCAAAAGAAAGGAAGCAAT |
| *Proteinase inhibitor-II* (*PI II*)[b] | Fw: GAAAATCGTTAATTTATCCCAC<br>Rev: ACATACAAACTTTCCATCTTTA |
| *SIEF* (Housekeeping gene)[b] | Fw: GATTGGTGGTATTGGAACTGTC<br>Rev: AGCTTCGTGGTGCATCTC |

§;Fw: forward, Rev: reverse, [a]Uppalapati *et al.* (2005); [b]Martínez-Medina *et al.* (2013).

[a]**Uppalapati SR, Ayoubi P, Weng H, Palmer DA, Mitchell RE, Jones W, Bender CL**. 2005. The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. The Plant Journal **42**, 201–217

[b]**Martínez-Medina A, Fernández I, Sánchez-Guzmán MJ, Jung SC, Pascual JA, Pozo MJ**. 2013. Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. Frontiers in Plant Science **4**, 1–12.

**Table S2 student *t-test* results on the performance of *Spodoptera exigua* feeding on *Meloidogyne incognita* infected plants.** *Spodoptera exigua* performance was assessed through larval weight gain, pupal weight, and pupation time. These performance indicators were measured from larvae feeding on leaves of control plants (without root infection) and leaves of plants infected in roots with *Meloidogyne incognita* (Mi) either at the invasion, galling, and reproduction stages. The data were analyzed for each performance indicator using student *t-test,* and statistically significant effects inferred at $p \leq 0.05$ and are indicated in bold.

| Performance indicator | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|
| | Df | T | P | Df | T | P | Df | T | P |
| Larval weight (mg) | 12 | -0.027 | 0.979 | 10 | 0.272 | 0.791 | 10 | -0.100 | 0.922 |
| Pupal weight (mg) | 16 | 0.327 | 0.748 | 28 | 1.986 | **0.057** | 15 | -0.079 | 0.938 |
| Pupation time (d) | 15 | 0.127 | 0.901 | 21 | -3.404 | **0.003** | 14 | 0.966 | 0.350 |

§; Df: degree of freedom, T: T-statistics value, P: probability value, mg: milligram, d: day(s).

**Table S3 ANOVA results on the concentrations of jasmonates in tomato leaves upon below and aboveground herbivory**. Concentrations of 12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA), and jasmonyl-*L*-isoleucine (JA-*Ile*) were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after *S. exigua* feeding. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Hormone | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P |
| OPDA | Mi | 1,29 | 1.9919 | 0.169 | 1,14 | 16.6384 | **0.001** | 1,14 | 2.2006 | 0.160 |
| | Se | 1,29 | 55.2256 | **<0.001** | 1,14 | 36.4689 | **<0.001** | 1,14 | 0.8498 | 0.372 |
| | Mi*Se | 1,29 | 2.1977 | 0.149 | 1,14 | 6.9356 | **0.020** | 1,14 | 0.2507 | 0.624 |
| JA | Mi | 1,30 | 6.7808 | **0.014** | 1,14 | 0.1724 | 0.684 | 1,15 | 3.3054 | 0.089 |
| | Se | 1,30 | 54.7066 | **<0.001** | 1,14 | 102.9905 | **<0.001** | 1,15 | 2.1413 | 0.164 |
| | Mi*Se | 1,30 | 7.7520 | **0.009** | 1,14 | 0.1278 | 0.726 | 1,15 | 3.1070 | 0.098 |
| JA-*Ile* | Mi | 1,28 | 0.7736 | 0.387 | 1,13 | 0.5259 | 0.481 | 1,15 | 3.2780 | 0.090 |
| | Se | 1,28 | 48.5152 | **<0.001** | 1,13 | 112.9965 | **<0.001** | 1,15 | 2.2171 | 0.157 |
| | Mi*Se | 1,28 | 0.6792 | 0.417 | 1,13 | 0.4483 | 0.515 | 1,15 | 2.2519 | 0.154 |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

**Table S4 ANOVA results on the expression of jasmonate-related defense marker genes in tomato leaves upon below and aboveground herbivory.** Transcript expression levels of *Lipoxygenase D (LoxD), Prosystemin (PS),* and *Proteinase inhibitor II (PI II)* were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after *S. exigua* feeding. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Marker genes | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P |
| *LoxD* | Mi | 1,31 | 35.359 | **<0.001** | 1,27 | 9.1802 | **0.005** | 1,23 | 0.5436 | 0.468 |
| | Se | 1,31 | 35.592 | **<0.001** | 1,27 | 334.0329 | **<0.001** | 1,23 | 29.1066 | **<0.001** |
| | Mi*Se | 1,31 | 33.397 | **<0.001** | 1,27 | 8.4198 | **0.007** | 1,23 | 0.6225 | 0.438 |
| *PS* | Mi | 1,28 | 10.8804 | **0.003** | 1,26 | 0.1394 | 0.712 | 1,26 | 0.3023 | 0.587 |
| | Se | 1,28 | 364.7633 | **<0.001** | 1,26 | 44.8585 | **<0.001** | 1,26 | 42.1531 | **<0.001** |
| | Mi*Se | 1,28 | 1.9591 | 0.173 | 1,26 | 0.0212 | 0.885 | 1,26 | 2.2918 | 0.142 |
| *PI II* | Mi | 1,29 | 13.902 | **0.001** | 1,24 | 0.9507 | 0.339 | 1,25 | 3.9052 | 0.059 |
| | Se | 1,29 | 38.258 | **<0.001** | 1,24 | 8.3204 | **0.008** | 1,25 | 14.7587 | **0.001** |
| | Mi*Se | 1,29 | 13.153 | **0.001** | 1,24 | 0.8216 | 0.374 | 1,25 | 3.6538 | 0.067 |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

**Table S5 ANOVA results on the trypsin protease inhibitor activity in tomato leaves upon below and aboveground herbivory.** The activity of trypsin proteases was determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 48 hours after *S. exigua* feeding. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected using Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Nematodes' infection stage | Source of variation | Statistics | | |
|---|---|---|---|---|
| | | $Df_{(n,d)}$ | F | P |
| Invasion | Mi | 1,16 | 0.0528 | 0.821 |
| | Se | 1,16 | 58.7901 | **<0.001** |
| | Mi*Se | 1,16 | 0.0763 | 0.786 |
| Galling | Mi | 1,16 | 0.1820 | 0.675 |
| | Se | 1,16 | 18.4215 | **0.001** |
| | Mi*Se | 1,16 | 0.0881 | 0.770 |
| Reproduction | Mi | 1,15 | 9.5735 | **0.007** |
| | Se | 1,15 | 44.5982 | **<0.001** |
| | Mi*Se | 1,15 | 10.2532 | **0.006** |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

**Table S6 ANOVA results on the concentrations of elemental carbon and nitrogen (in percentages), and carbon/nitrogen ratio in tomato leaves upon below and aboveground herbivory.** The concentrations of carbon (C), nitrogen (N), and carbon/nitrogen (C/N) ratio were determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after *S. exigua* feeding. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Parameter | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P | $Df_{(n,d)}$ | F | P |
| C | Mi | 1,29 | 1.3330 | 0.258 | 1,32 | 0.1946 | 0.662 | 1,32 | 0.1474 | 0.704 |
| | Se | 1,29 | 1.2932 | 0.265 | 1,32 | 0.0000 | 1.000 | 1,32 | 0.2192 | 0.649 |
| | Mi*Se | 1,29 | 0.0003 | 0.987 | 1,32 | 0.0015 | 0.970 | 1,32 | 0.2086 | 0.651 |
| N | Mi | 1,30 | 0.4979 | 0.486 | 1,29 | 2.4423 | 0.129 | 1,31 | 0.5371 | 0.469 |
| | Se | 1,30 | 0.3763 | 0.544 | 1,29 | 0.0196 | 0.890 | 1,31 | 0.4650 | 0.500 |
| | Mi*Se | 1,30 | 1.0931 | 0.304 | 1,29 | 0.2722 | 0.607 | 1,31 | 0.0083 | 0.928 |
| C/N ratio | Mi | 1,30 | 0.6306 | 0.433 | 1,31 | 6.1619 | **0.019** | 1,30 | 0.0025 | 0.960 |
| | Se | 1,30 | 0.7389 | 0.397 | 1,31 | 0.0376 | 0.847 | 1,30 | 0.0001 | 0.994 |
| | Mi*Se | 1,30 | 0.7231 | 0.402 | 1,31 | 0.9819 | 0.329 | 1,30 | 0.1185 | 0.733 |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

**Table S7 ANOVA results on the LC-MS intensities of the selected metabolites in tomato leaves upon below and aboveground herbivory.** LC-MS of the selected metabolites were determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed at the nematodes' galling stage. Samples were taken 24 hours after *S. exigua* feeding. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| mass to charge ratio (*m*/*z*), retention time (rt) in minutes | Predicted metabolites | Source of variation | Galling | | |
|---|---|---|---|---|---|
| | | | $Df_{(n,d)}$ | F | P |
| *m*/*z* 203.053, rt 0.93 | Polyamine conjugated to a phenylpropanoid | Mi | 1,11 | 71.523 | **<0.001** |
| | | Se | 1,11 | 107.425 | **<0.001** |
| | | Mi*Se | 1,11 | 10.194 | **0.009** |
| *m*/*z* 576.389, rt 5.74 | α-dehydrotomatine | Mi | 1,14 | 0.8967 | 0.360 |
| | | Se | 1,14 | 1.6044 | 0.226 |
| | | Mi*Se | 1,14 | 1.0969 | 0.313 |
| *m*/*z* 578.4056, rt 6.03 | α-tomatine | Mi | 1,12 | 1.0117 | 0.334 |
| | | Se | 1,12 | 8.5875 | **0.013** |
| | | Mi*Se | 1,12 | 2.6976 | 0.126 |
| *m*/*z* 188.0707, rt 3.44 | Unknown | Mi | 1,13 | 1.0675 | 0.320 |
| | | Se | 1,13 | 313.3833 | **<0.001** |
| | | Mi*Se | 1,13 | 4.6465 | **0.050** |
| *m*/*z* 348.187, rt 4.5 | Unknown | Mi | 1,13 | 15.731 | **0.007** |
| | | Se | 1,13 | 330.364 | **<0.001** |
| | | Mi*Se | 1,13 | 23.491 | **0.000** |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

# CHAPTER 3

# The impact of *Spodoptera exigua* herbivory on *Meloidogyne incognita* induced root responses depends on the nematodes' life cycle stages

AoB PLANTS 2020, Vol. 12, No. 4

doi:10.1093/aobpla/plaa029
Advance Access Publication June 24, 2020
Studies

STUDIES

# The impact of *Spodoptera exigua* herbivory on *Meloidogyne incognita*-induced root responses depends on the nematodes' life cycle stages

Crispus M. Mbaluto[1,2], Esraa M. Ahmad[3], Melody Fu[4], Ainhoa Martínez-Medina[1,5] and Nicole M. van Dam[1,2,*]

[1]Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany, [2]Institute of Biodiversity, Friedrich-Schiller-Universität-Jena, Dornburger Str. 159, 07743 Jena, Germany, [3]Department of Genetics, Faculty of Agriculture, Cairo University, Cairo University Road, Giza 12613, Egypt, [4]Faculty of Land and Food Systems, University of British Columbia, 2357 Main Hall Vancouver, BC V6T 1Z4, Canada, [5]Plant-Microorganism Interaction Unit, Institute of Natural Resources and Agrobiology of Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain

*Corresponding author's e-mail address: nicole.vandam@idiv.de

**Associate Editor:** Shahid Siddique

**Citation**: Mbaluto CM, Ahmad EM, Fu M, Martínez-Medina A, van Dam NM. 2020. The impact of *Spodoptera exigua* herbivory on *Meloidogyne incognita*-induced root responses depends on the nematodes' life cycle stages. *AoB PLANTS* **12**: plaa029; doi: 10.1093/aobpla/plaa029

## Abstract

Induced responses to above-ground and below-ground herbivores may interact via systemic signalling in plants. We investigated whether the impact of above-ground herbivory on root-knot nematode-induced responses depends on the nematode's life cycle stages. Tomato plants were infected with the nematode (*Meloidogyne incognita*) for 5, 15 or 30 days before receiving *Spodoptera exigua* caterpillars above-ground. We collected root materials after 24 h of caterpillar feeding. We investigated phytohormones and α-tomatine levels, and the expression of defence and glycoalkaloid metabolism (GAME) marker genes in tomato roots. Nematode infection alone increased the endogenous root levels of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), α-tomatine and the expression of the *GLYCOALKALOID METABOLISM 1 (GAME1)* gene mostly at 30 days post-nematode inoculation. Caterpillar feeding alone upregulated *Lipoxygenase D* and downregulated *Basic-β-1-glucanase* and *GAME1* expression in roots. On nematode-infected plants, caterpillar feeding decreased JA levels, but it increased the expression of *Leucine aminopeptidase A*. The induction patterns of ABA and SA suggest that caterpillars cause cross-talk between the JA-signalling pathway and the SA and ABA pathways. Our results show that caterpillar feeding attenuated the induction of the JA pathway triggered by nematodes, mostly in the nematodes' reproduction stage. These results generate a better understanding of the molecular and chemical mechanisms underlying frequent nematode–plant–caterpillar interactions in natural and agricultural ecosystems.

**Keywords:** Above-ground–below-ground interaction; phytohormones; root-knot nematodes; *Solanum lycopersicum*; *Spodoptera exigua*; steroidal glycoalkaloids; systemic-induced responses.

**Received:** 19 February 2020; **Editorial decision:** 1 June 2020; **Accepted:** 17 June 2020

## Introduction

Tomato is ranked the most consumed vegetable globally, with >170.8 million tons produced in 2017 alone (Omondi 2018; FAO 2019). This yield is ~30 % times more than a decade earlier (Oishimaya 2017). Like other crops, tomato plants experience high pest pressure by, e.g., nematodes, arthropods, bacterial and fungal pathogens. This pest pressure reduces the growth and limits tomato yield (Kumar *et al.* 2016; Garcia *et al.* 2018; van Dam *et al.* 2018). Root-knot nematodes (RKNs) are globally occurring, soil-borne pathogens that attack plants at their roots. The infective second-stage juveniles (J2s) hatch in the soil, where they locate and infect the roots of a susceptible host. Upon penetrating the roots, the J2s migrate intercellularly until they reach the vascular tissues. There they establish their permanent feeding sites (Niebel *et al.* 1994; Williamson and Gleason 2003; Gheysen and Mitchum 2011). Their infection impairs the translocation of water and minerals from the roots to the shoots, which can limit the plant's productivity and fitness (Abad *et al.* 2008; Jones *et al.* 2013). At the same time, above-ground (AG) herbivores, such as leaf-chewing caterpillars, may be present on the plant. The leaf loss due to caterpillar feeding can adversely impact on primary plant processes, such as the rate of photosynthesis, which are directly related to the plant's productivity (Meyer and Whitlow 1992; Mitchell *et al.* 2016). Together the damage caused by RKN and herbivorous insects can reduce crop production by ~20 % annually, making them agro-economically important crop pests (Karajeh 2008; van der Meijden 2015; Mitchell *et al.* 2016). Commonly, chemical pesticides are used to control crop pests, such as nematodes and insect herbivores. Although these pesticides might be effective, several of them are currently banned from use due to their detrimental effects on human health and the environment (Franco *et al.* 2015; Borel 2017). Efforts to identify natural plant resistance traits for AG and below-ground (BG) herbivores may help to develop sustainable pest management strategies.

Plants rely on constitutive and inducible defence responses to protect themselves against attackers. Constitutive responses are described as the physical barriers, such as thorns and trichomes, and chemical traits, such as alkaloids and glucosinolates, usually expressed independently of herbivore or pathogen attack (Wittstock and Gershenzon 2002). Induced defences are stimulated by herbivore feeding or pathogen attack, which results in the induction of specific plant phenotypic responses (Karban 2011; Boots and Best 2018). In addition, plants can tolerate herbivory via the re-allocation of resources to undamaged plant parts, followed by compensatory growth, or by increasing the rate of photosynthesis (Mauricio *et al.* 1997; Peterson *et al.* 1998; Retuerto *et al.* 2004; Boege *et al.* 2007; Núñez-Farfán *et al.* 2007; Fornoni 2011; Koch *et al.* 2016; Mitchell *et al.* 2016). These changes influence critical plant physiological processes and can adversely impact the performance of herbivores.

Plant hormonal signalling governs herbivore-induced defence responses. Among the many plant hormones described so far, jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) are the main signalling hormones that fine-tune plant defence responses upon attack (Pieterse *et al.* 2009, 2012). Interaction, or cross-talk, between phytohormonal pathways, results in specific defence responses, which tailor the defensive response to the particular attacker (Pieterse *et al.* 2009, 2012; Li *et al.* 2019). Induction of defence responses at the site of attack often results in systemic signalling to distal non-attacked plant parts, thereby protecting them against future attacks (Martínez-Medina *et al.* 2013; van Dam *et al.* 2018). Moreover, systemic-induced responses may influence the attraction, behaviour and performance of other organisms sharing the same host (Bruce 2014). As a consequence, induced responses play an essential role in indirect interactions between AG and BG herbivores feeding on the same plant (van Dam and Heil 2011).

Most studies investigating plant-mediated interactions between AG and BG herbivores focus on how AG herbivore-induced responses are affected by BG herbivory (Erb *et al.* 2009; Kumar *et al.* 2016; Arce *et al.* 2017; Hoysted *et al.* 2017; van Dam *et al.* 2018). Only a few studies analysed how AG-induced responses affect BG-feeding herbivores or pathogens. These studies report that AG herbivory induces systemic responses in the roots of crops (e.g., potato, tomato) and grass species (Kafle *et al.* 2017; Wang *et al.* 2017; Hoysted *et al.* 2018). Both primary and secondary metabolites play a role in plant-mediated interactions between AG and BG insect herbivores. For example, AG feeding by aphids changes potato root exudates by reducing amounts of glucose and fructose, which diminish cyst hatching (Hoysted *et al.* 2018). Defoliation by clipping increases nitrogen concentration in roots, which in return increases the total abundance of two species of root-feeding nematodes (Wang *et al.* 2017). Similarly, AG feeding by *Manduca sexta* on *Nicotiana attenuata* induces jasmonate-dependent facilitation of plant-parasitic nematode (PPN) abundance in the field, and RKN (*Meloidogyne incognita*) reproduction in a greenhouse (Machado *et al.* 2018). Collectively, these studies demonstrate that plant responses induced by AG herbivory can systemically affect BG defence responses.

The few studies available show that systemic-induced responses triggered by AG herbivory cause different effects on root feeders (Huang *et al.* 2013; Kafle *et al.* 2017; Wang *et al.* 2017; Hoysted *et al.* 2018; Machado *et al.* 2018). Partly the differences in the observed interaction outcomes are due to variation in the timing and sequence of arrival of both AG- and BG-feeding organisms (Erb *et al.* 2011; Kafle *et al.* 2017; Wang *et al.* 2017). In nature, root herbivores commonly colonize the plant before shoot herbivores arrive. This natural sequence of pest arrival follows from the fact that roots develop first (Bezemer and van Dam 2005). For PPNs, such as RKNs, these factors are particularly relevant. As obligate root feeders, RKNs undergo different distinct life cycle stages. In the *invasion stage*, J2s enter the root at the zone of elongation and move towards the vascular cylinder. Then they turn around and move several body lengths upwards before settling and initiating feeding (Robinson and Perry 2006). This movement occurs between the cells (intercellularly), which also reduces the elicitation of defence responses because only a few cells are damaged (Caillaud *et al.* 2008; Gheysen and Mitchum 2011). In the *establishment stage*, the juveniles become sedentary and inject various effectors to establish the so-called 'giant cell'. This giant cell serves as their feeding site. The cells surrounding the giant cells undergo proliferation and enlargement, and, in due time, they become visible to the human eye as a gall or a 'root-knot' (Rodiuc *et al.* 2014; Escobar *et al.* 2015). We refer to this stage, in which the nematode establishes a feeding site, as the galling stage. At their feeding site, the nematodes acquire resources and develop through three molts to mature and reach the *reproduction stage*. The female nematode's body swells up and becomes pear-shaped. When the eggs are ripe, the females release their eggs into the rhizosphere, and another cycle begins (Caillaud *et al.* 2008; Gheysen and Mitchum 2011). In each infection stage, the nematodes' growth and development depend on the injection of different effectors into the host cells (Quentin *et al.* 2013;

Favery *et al.* 2016; Gheysen and Mitchum 2019). These effectors trigger different hormonal signalling pathways, including JA, SA, ET and ABA (Caillaud *et al.* 2008; Kyndt *et al.* 2017; Gheysen and Mitchum 2019). Because hormones are generally involved in plant defence induction, systemic defence responses induced by AG herbivores might affect nematodes and the local responses they induce in the roots. Moreover, the effect that AG herbivores may have on BG defence signalling triggered by root herbivores may depend on the life cycle stage in which the nematodes are at the time point of AG attack.

Here, we used tomato (*Solanum lycopersicum* 'Moneymaker') and two generalist crop pests, the RKN *M. incognita* and larvae of *Spodoptera exigua*, as the study system to analyse the molecular mechanisms mediating interactions between AG herbivores and nematodes. Previous studies showed that interactions between RKN and shoot herbivores can be governed by JA-dependent responses, evidenced by changes in jasmonates levels in *N. attenuata* (Machado *et al.* 2018) and the production of trypsin protease inhibitors in tomato (Arce *et al.* 2017). These interactions may also involve cross-talk between hormonal pathways, such as JA–SA (van Dam *et al.* 2018) and JA–ABA (Erb *et al.* 2009; Kyndt *et al.* 2017). Therefore, we measured phytohormone concentrations (JA, SA, ABA) and the expression of several marker genes for hormonal signalling; *Lipoxygenase D* and *Leucine aminopeptidase A* (JA markers), *Le4* (ABA marker) and *Basic-β-1,3-glucanase (GluB)* (ET marker) in roots **[see Supporting Information—Table S1]**. Tomato is also known to produce steroidal glycoalkaloids, such as α-tomatine, as a defence to generalist herbivores (Friedman 2002; Cárdenas *et al.* 2015). Hence, we included measurements of α-tomatine and the expression of glycoalkaloid metabolism (GAME) genes *Jasmonate-responsive Ethylene Response Factor 4 (JRE4)* and *GAME1*. We specifically analysed how 24 h of AG feeding affected these defence-related traits in roots that were infected with *M. incognita* at 5, 15 and 30 days post-nematode inoculation (dpi). These time points coincide with the invasion (5 dpi), galling (15 dpi) and reproduction (30 dpi) stages of this nematode. With this approach, we aimed to assess whether the nature of the interaction between shoot- and root-induced responses depends on the developmental stage of the RKN.

## Materials and Methods

### Study plant, root and shoot organisms

In all our experiments, we used tomato (*S. lycopersicum* 'Moneymaker') as the model plant. The RKN *M. incognita* was used as root herbivore, and the larvae of the generalist herbivore *S. exigua* were used as shoot herbivores. We obtained *M. incognita* eggs from Rijk Zwaan (De Lier, The Netherlands) and maintained a glasshouse stock on tomato 'Moneymaker' for 8 weeks. Similar to a previous study (Martínez-Medina *et al.* 2017), we initiated the colony from a single egg mass, and 8 weeks later extracted eggs for use in the bioassay. We purchased *S. exigua* eggs from Entocare C.V. Biologische Gewasbescherming (Wageningen, The Netherlands) and maintained a laboratory colony on artificial diet, in a growth chamber set at 25 °C constant, 12-h photoperiod and 45 % relative humidity (RH).

### Plant growth condition and herbivores infection

The tomato seeds were obtained from Intratuin B.V (Woerden, The Netherlands). Before germination, the seeds were surface-sterilized by immersion in 40 mL of 10 % sodium hypochlorite solution for 4 min. Afterward, the seeds were rinsed four times with water. Each round of rinsing was for 10 min. The sterilized seeds were placed on moistened glass beads and allowed to germinate at 27 °C in the dark for 3 days, followed by 4 days in a plant growth chamber (CLF PlantClimatic, CLF PlantClimatics GmbH, Wertingen, Germany). The growth conditions were 16-h:8-h day:night cycle, 55 % RH and 60 % (65 μmol $s^{-1}$ $m^{-2}$) light intensity. One-week-old seedlings were transplanted into sterilized 1:1 sand:soil mixture in 11 × 11 × 12 cm pots. They were grown in a glasshouse at 26 ± 3 °C:23 ± 3 °C day:night, with 16-h:8-h light:dark and RH was maintained at ~30 %. The plants were watered as required and supplemented weekly with 50 % strength Hoagland solution. The plants were grown for three more weeks before using them in bioassays. We randomly selected healthy plants of similar size and appearance for our experimental treatments. We divided the plants into two groups; one group was inoculated with *M. incognita* eggs (3000 eggs per mL), and the other group was mock-inoculated with water. In the *M. incognita*-inoculated plants, we set three time points to coincide with the main nematode life cycle stages. These were 5 dpi (invasion stage), 15 dpi (galling stage) and 30 dpi (reproduction stage). At each of these time points, plants were subjected to four different treatments, each with 10 biological replicates. The treatments were control (plants without herbivores or nematodes); BG infection (plants challenged with *M. incognita*); AG herbivory (plants challenged with *S. exigua*); and both BG infection and AG herbivory (plant challenged with *M. incognita* in the roots followed by *S. exigua* feeding on leaves). We infested the plants assigned to leaf feeding with one second-instar *S. exigua* caterpillar. The *S. exigua* caterpillars were confined to a 7-cm (diameter) round clip cage placed on one fully expanded leaf close to the tip (see Fig. 4D in Bandoly and Steppuhn (2016)). In plants without shoot herbivory, an empty clip cage was mounted on a leaf at a similar position to the one used in plants with shoot herbivory. The *S. exigua* larvae were allowed to feed for 24 h. Other studies showed that this time period suffices to affect defence metabolites and genes in roots. For example, 24 h of AG herbivory by *M. sexta* and *Spodoptera littoralis* on *N. attenuata* results in systemic induction of JA-related genes expression in roots (Fragoso *et al.* 2014). After this time, we harvested the roots by gently removing them from the pots. The soil was removed by soaking the whole root into a bucket filled with tap water. Then the roots were rinsed with running tap water and dried with filter paper. After quickly counting the number of galls (especially for roots collected at the galling and reproduction stages) **[see Supporting Information—Fig. S1]**, the roots were wrapped in clean labelled aluminium foil, and immediately shock-frozen in liquid nitrogen. The root samples were stored at –80 °C, pending molecular and metabolite analyses.

### Quantitative reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from ~100 mg fresh weight per root sample according to the method described by Oñate-Sánchez and Vicente-Carbajosa (2008). First-strand cDNA was synthesized from 1 μg DNase-free mRNA using Revert Aid H-minus RT (Thermo Fisher Scientific Baltic UAB, Vilnius, Lithuania) following the manufacturer's instructions. Real-time qPCR reactions and relative quantification of specific mRNA levels were performed according to Martínez-Medina *et al.* (2017) by using a CFX 384 Real-Time PCR system (Bio-Rad Laboratories Inc., Singapore) and the gene-specific primers described in **Supporting Information—Table S1**. These genes were selected from previously published articles where their involvement in

tomato biotic interactions is reported (Uppalapati *et al.* 2005; Martínez-Medina *et al.* 2013; Yan *et al.* 2013; Abdelkareem *et al.* 2017). The data were normalized using the housekeeping gene (*SlEF X14449*), which encodes for the tomato elongation factor-1α, a commonly used and stable reference gene for data normalization in studies on induced responses in tomato (Miranda *et al.* 2013; Martínez-Medina *et al.* 2017). Data were analysed by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

## Determination of phytohormone concentration

We extracted and quantified phytohormones following the protocol described by Machado *et al.* (2013). In brief, ~100 mg fresh weight per root sample was extracted with 1 mL ethyl acetate containing 40 ng of each of the following internal phytohormone standards: $D_6$-JA and $D_6$-SA, and $D_6$-ABA. The extracts were vortexed for 10 min using a Thermomixer, then centrifuged at 15 000 × g, 4 °C for 20 min, the supernatants were transferred to a new tube and evaporated to dryness at room temperature using a SpeedVac (Labconco Co-operation, Kansas, MO, USA). Remaining pellets were resuspended in 200 µL methanol:water (70:30) using an ultrasonic bath for 5 min and centrifuged at 15 000 × g, 4 °C for 5 min. The supernatant was collected for phytohormone measurement using liquid chromatography (Bruker Advance UHPLC, Bremen, Germany) coupled to a mass spectrometer (Bruker Elite EvoQ Triple quadrupole, Bremen, Germany) (LC/MS EVOQ) (Schäfer *et al.* 2016). The separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 µm, 80 Å, Agilent technologies, Santa Clara, CA, USA). Mobile phase was composed of A (0.05 % (v/v) aqueous formic acid) and B (0.05 % (v/v) formic acid in 100 % acetonitrile). The following gradient was used: 0–0.5 min, 5 % B; 0.5–0.6 min, 5–50 % B; 0.6–2.5 min, 50–100 % B; 2.5–3.5 min, 100 % B; 3.5–3.55 min, 100–5 % B; 3.55–4.5 min, 5 % B at flow rate of 400 µL $min^{-1}$. All solvents used were LC-MS grade. The column temperature was kept constant at 42 °C.

After separation, the compounds were nebulized by electron spray ionization in the negative mode using the following conditions: capillary voltage 4500 eV, cone gas 35 arbitrary units/350 °C, probe gas 60 arbitrary units/475 °C and nebulizing gas at 60 arbitrary units. The phytohormones were identified based on their retention time and the monitored mass to charge ratio (*m*/*z*) transition. The *m*/*z* ratio of the phytohormones of interest were; (*m*/*z*) 209.12 → 59.00 for JA; (*m*/*z*) 263.13 → 153.00 for ABA and (*m*/*z*) 137.02 → 93.00 for SA. Samples were analysed in a randomized sequence with acetonitrile samples in between as background controls. Data acquisition and processing were performed using the 'MS data Review' software (Bruker MS Workstation, version 8.2). Phytohormone levels were calculated based on the peak area of the corresponding internal standard and the amount of fresh mass of plant material ($ng^{-1}$ $mg^{-1}$ fresh weight).

## Determination of the root α-tomatine concentrations

We extracted ~100 mg fresh weight of each root sample in a 2-mL Eppendorf tube with 1 mL solution containing 25 % of acetate buffer (2.3 mL acetic acid, 3.41 mg ammonium acetate dissolved in 1 L of Milli pure water, pH 4.8) and 75 % methanol. Tubes with extracts were inverted for 10 s and thoroughly mixed via shaking using a grinding ball mill (MM400, Retsch GmbH, Leipzig, Germany) set at 30 Hz for 5 min. To remove the solid particles in the extracts, we centrifuged them at 15 000 × g for 15 min, and the supernatant transferred into a new 2-mL Eppendorf tube, and the pellet was re-extracted as above. We mixed the first and second supernatant and transferred 200 µL of the combined extracts into a 2-mL HPLC vial and added 800 µL of the extraction buffer to obtain a 1:5 dilution for each sample. The extracts were stored at –20 °C, pending further analysis. Metabolites were characterized by injecting 1 µL of the extracts in a UPLC (Dionex 3000, Thermo Scientific). The chromatograph was equipped with a C18 column (Acclaim TM RSLC 120), 2.1 × 150 mm external dimension, 2.2 µm particle size and 120 Å pore size. The column was kept at 40 °C. The mobile phases (LC-MS grade solvents) were composed of solvent A: 0.05 % (v/v) aqueous formic acid and solvent B: 0.05 % (v/v) formic acid in acetonitrile. The multi-step gradient for solvent B was; 0–1 min 5 %, 1–4 min 28 %, 4–10 min 36 %, 10–12 min 95 %, 12–14 min 95 %, 14–16 min 5 %, 16–18 min 5 %. The flow was set to 400 µL $min^{-1}$. We detected compounds using a maXis impact HD MS-qToF (Bruker Daltonics). Data were acquired in positive mode. Electron Spray Ionisation ion source conditions were; endplate offset = 500 V, capillary = 4500 V, nebulizer = 2.5 bar, dry gas = 11 L $min^{-1}$, dry temperature = 220 °C. Transfer line conditions were: funnels 1 and 2 = RF 300 Vpp, isCID energy = 0 eV, hexapole = 60 Vpp, quadrupole ion energy = 5 eV, low mass = 50 *m*/*z*, collision cell energy = 10 eV, collision RF 500 Vpp, transfer time = 60 µs, pre-pulse storage = 5 µs. The mass spectrometer operated with a mass range of 50–1500 *m*/*z* and a spectral acquisition rate of 3 Hz. Sodium formate clusters (10 mM) were used for calibrating the *m*/*z* values. These sodium formate clusters were a mix consisting of 250 mL isopropanol, 1 mL formic acid, 5 mL 1 M NaOH and the final volume was adjusted to 500 mL. All analyses had a quality control sample, which was a pool of all the different experimental groups and time points. The quality control sample was analysed at the beginning and the end of the batch and after every 10 injections. The raw data files were processed using the program Compass DataAnalysis (Bruker Daltonics). The processing involved obtaining the extracted ion chromatogram (EIC) for a fragment of α-tomatine at the *m*/*z* value 578.4050 and *m*/*z* tolerance of ±0.1. We selected the option compound list to automatically calculate the peak areas of each EIC per sample per study time point. All the peak areas for α-tomatine were tabulated and used for multivariate statistical analysis.

## Statistical analysis

We created two data sets combining (i) phytohormone and α-tomatine levels, and (ii) defence markers and glycoalkaloid metabolism genes. In each combined data set, we tested the effects of *M. incognita* (Mi; with vs. without), and *S. exigua* (Se; with vs. without), and their interactions on the defence variables (i.e. the plant defence traits; phytohormone, α-tomatine and marker genes). Each data set was analysed using the permutational multivariate analysis of variance (PERMANOVA). Permutational multivariate analysis of variance was chosen because our data lacked homogeneity of variance or normal distribution; PERMANOVA does not require this because it uses a distribution-free permutation approach to partition the variance among treatments (Anderson 2017). The PERMANOVA analysis was run for each data set using the Adonis function, with the Gower dissimilarities method among samples, and 999 permutations in R v 3.6.1 software (R Core Development Team 2019). Where the PERMANOVA output showed significant effects for either factor or their interaction **[see Supporting Information—Tables S2 and S4]**, we performed separate factorial linear model ANOVAs on each dependent variable, with *M. incognita* and *S. exigua* and their interaction as fixed factors. Once the main effect significantly affected any of the dependent variables, the differences among the four

experimental treatments were tested using Tukey's Honest Significant Difference test for multiple comparisons.

## Results

### Root infection by *M. incognita* alone affects the expression of root-inducible defences at different life cycle stages

We first considered how the nematode affected root-inducible defences at the invasion, galling and reproduction stages. We found that *M. incognita* root infection enhanced the induction of JA, SA, ABA and α-tomatine progressively during the infection process. In particular, the JA response in *M. incognita*-infected plants became more pronounced with the progression of the nematode's life cycle compared to controls (Fig. 1A, E and I, blue box plots). At both the invasion and galling stages, the levels of these signalling hormones were increased, but only at the reproduction stage, the increases became significant compared to control plants (Fig. 1I–L, blue box plots; **see Supporting Information—Table S3**). In contrast, root infection by *M. incognita* did not trigger changes in the expression of the defence marker genes. We found that the expression of *LoxD*, *LapA*, *Le4*, *GluB* (Fig. 2, blue box plots; **see Supporting Information—Table S5**) and *JRE4* (Fig. 3, blue box plots; **see Supporting Information—Table S5**) remained similar to those observed in control plants regardless of the nematodes' root infection stage. However, we observed significant upregulation in the expression of the *GAME1* transcripts at the nematodes' reproduction stage compared to control plants (Fig. 3F, blue box plot; **see Supporting Information—Table S5**). The increase in *GAME1* transcripts correlated with an increase in α-tomatine concentrations in nematode-infected roots at the reproduction stage (Fig. 1L, blue box plot).

### The impact of *S. exigua* feeding on root defence responses in tomato plants depends on plant age

Next, we analysed the impact of *S. exigua* leaf herbivory on root defences of tomato plants without nematode infection. Due to the experimental set-up, which was designed based on the life stages of the nematodes, the plants that received only caterpillars were 4.8 (coinciding with the invasion stage), 6.2 (coinciding with the galling stage) and 8 (coinciding with reproduction stage) weeks old. We found that *S. exigua* leaf herbivory did not affect the levels of JA, SA, ABA and α-tomatine in tomato roots compared to the control plants, regardless of plant age (Fig. 1, green box plots; **see Supporting Information—Table S3**). In contrast, *S. exigua* herbivory triggered differential effects on the expression of the hormonal signalling and GAME marker genes (Figs 2 and 3, green box plots; **see Supporting Information—Table S5**). In the 4.8 (invasion stage) and 6.2 (galling stage) weeks old plants, the expression of the marker genes

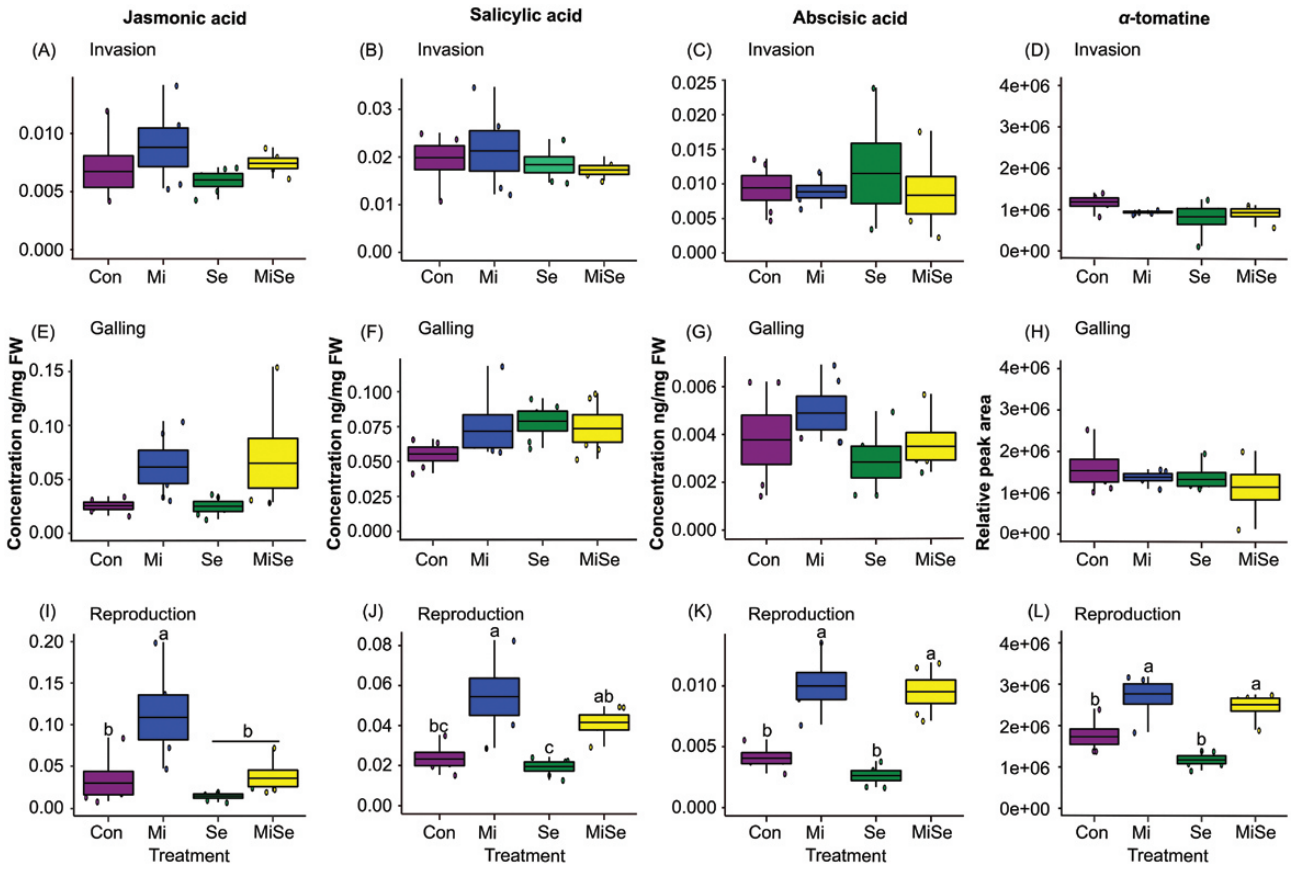


**Figure 1.** Phytohormone concentrations and relative peak area of α-tomatine. Mean concentrations (ng mg$^{-1}$ fresh weight) of phytohormones and the relative peak area of α-tomatine in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plant without herbivory. Box plots are the mean (±SEM) of jasmonic acid (A, E, I); salicylic acid (B, F, J); abscisic acid (C, G, K); α-tomatine (D, H, L) per treatment ($n$ = 5) measured at the nematodes' invasion (A–D), galling (E–H) and reproduction (I–L) stages. Different lower-case letters above the box plots indicate significant differences in mean values between treatments, determined via multiple comparisons Tukey's HSD test after ANOVA at $P \leq 0.05$.

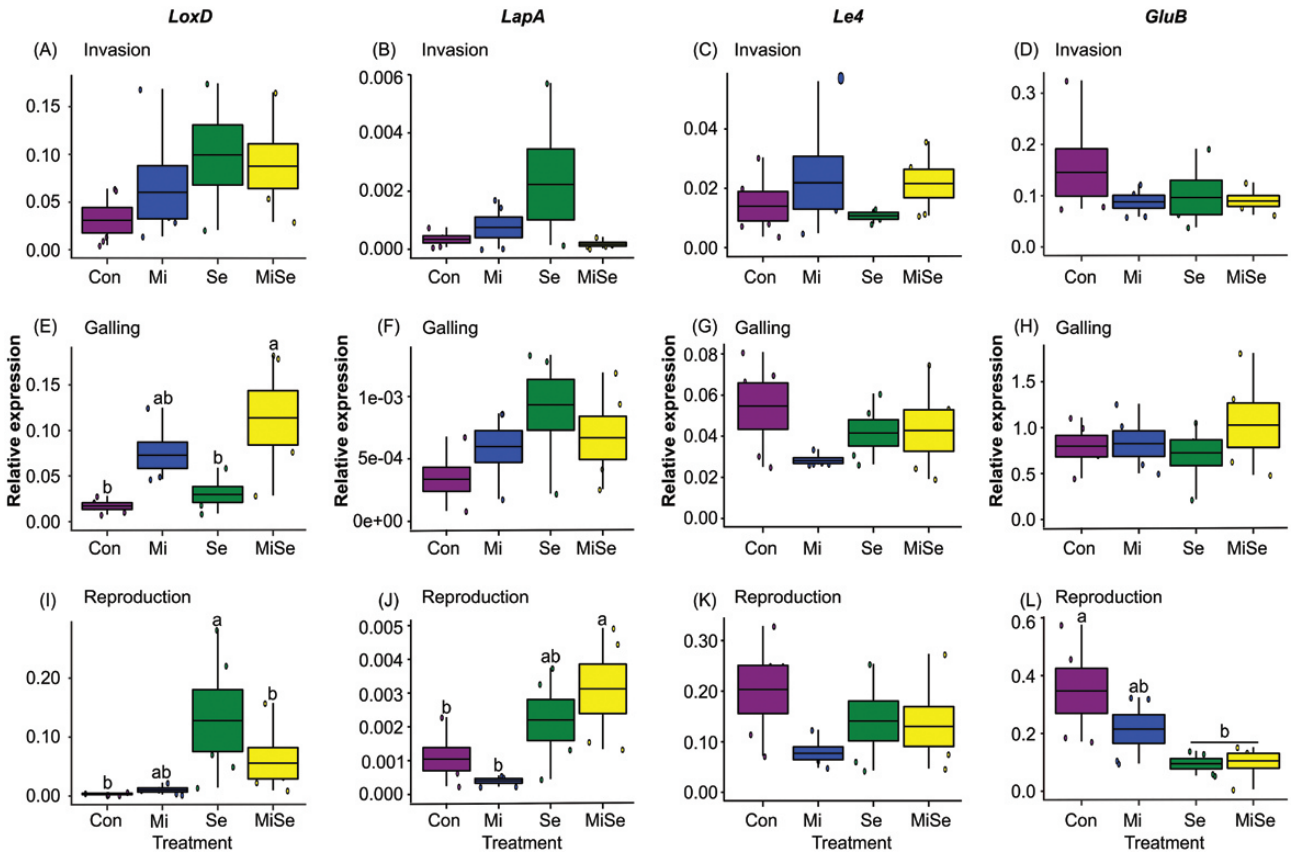


**Figure 2.** Expression of defence marker genes. Relative expression of defence marker genes in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plants without herbivory. Expression values are normalized over the expression of the housekeeping gene (*SlEF X14449*) encoding for tomato elongation factor-1α. Box plots are mean (±SEM) expression values of *Lipoxygenase D* (*LoxD*); *Leucine aminopeptidase A* (*LapA*); *abscisic acid-responsive Le4* (*Le4*); *Basic-β-1-glucanase* (*GluB*) per treatment ($n = 5$) measured at the nematodes' invasion (A–D), galling (E–H) and reproduction (I–L) stages, respectively. Different lower-case letters above the box plots indicate significant differences in mean expression among treatments, determined via multiple comparisons Tukey's HSD test after ANOVA at $P \leq 0.05$.

was not significantly different from controls (Figs 2A–H and 3A–D, green box plots; **see Supporting Information—Table S5**). Notably, when the plants were 8 weeks old, which coincided with the nematodes' reproduction stage, the defence gene *LoxD* was upregulated compared to controls (Fig. 2I, green box plot; **see Supporting Information—Table S5**). The *LapA* and *Le4* expression levels were not significantly different compared to controls (Fig. 2J and K, green box plots; **see Supporting Information—Table S5**), while *GluB* was significantly downregulated compared to controls (Fig. 2L, green box plot; **see Supporting Information—Table S5**). The GAME gene *JRE4* was not affected while the *GAME1* was significantly downregulated compared to controls (Fig. 3E and F, green box plots; **see Supporting Information—Table S5**).

## Effects of *S. exigua* on *M. incognita*-induced responses depend on the nematodes' infection stage

Because our primary interest was to analyse the effect of *S. exigua* AG feeding on root responses induced by *M. incognita* at different infection stages, we primarily focused on the comparison between *M. incognita*-infected plants (Mi treatment, blue box plots, Figs 1–3) with the double-infected plants (MiSe treatment, yellow box plots, Figs 1–3). We found that *S. exigua* herbivory on *M. incognita*-infected plants did not change JA levels at the invasion and galling stages compared to plants challenged with *M. incognita* alone (Fig. 1A and E, yellow box plots; **see Supporting Information—Table S3**). *Spodoptera exigua* herbivory on the *M. incognita*-infected plants significantly decreased the JA levels at the nematodes' reproduction stage compared to plants infected with *M. incognita* alone (Fig. 1I, yellow box plot; **see Supporting Information—Table S3**). *Spodoptera exigua* feeding on *M. incognita*-infected plants did not affect SA, ABA and α-tomatine concentrations compared to plants challenged with *M. incognita* alone, regardless of the nematodes' infection stage (Fig. 1B–D, F–H and J–L, yellow box plots; **see Supporting Information—Table S3**). Overall, we observed that the local nematode-induced responses dominated the nature of SA, ABA and glycoalkaloid responses in roots (Fig. 1; **see Supporting Information—Table S3**, main Mi effects). Similarly, *S. exigua* herbivory on *M. incognita*-infected plants triggered changes in the expression of marker genes depending on the nematodes' root infection stages. At the invasion stage, the expression levels of both defence and GAME genes in double-infected plants were similar to those with *M. incognita* infection alone (Figs 2A–D, and 3A and B, yellow box plots; **see Supporting Information—Table S5**). At the galling stage, the JA biosynthesis marker *LoxD* overall increased in plants infected with *M. incognita* (**see Supporting Information—Table S5**, main Mi effect). Above-ground damage by *S. exigua* did not significantly alter this. A similar pattern was found for the expression levels of the other marker genes in plants with *M. incognita* and *S. exigua*; in the invasion and galling stage their expression levels were similar to plants with

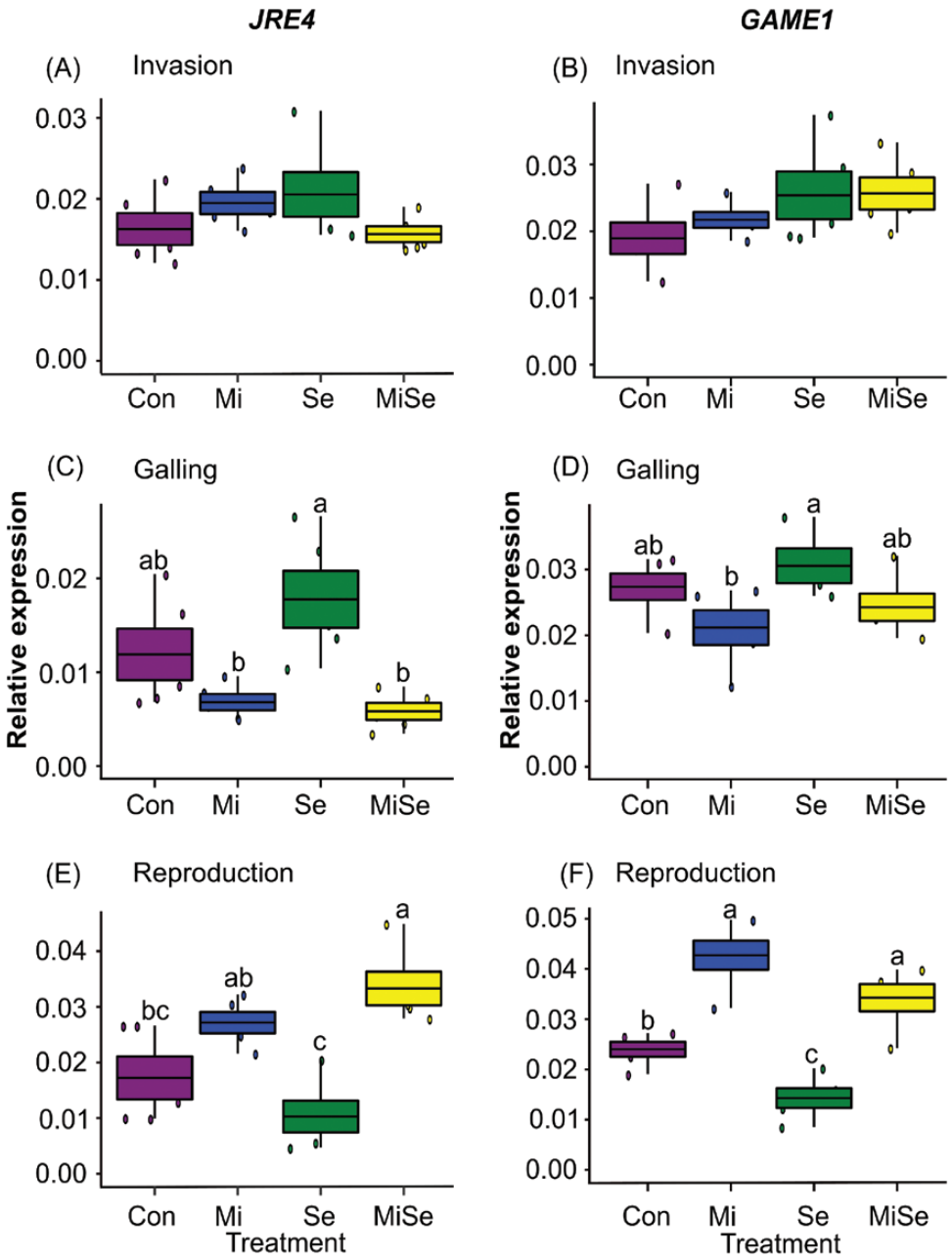


**Figure 3.** Expression of steroidal glycoalkaloid metabolism genes. Relative expression of steroidal glycoalkaloid metabolism genes in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plants without herbivory. Expression values are normalized over the expression of the housekeeping gene (*SlEF X14449*) encoding for tomato elongation factor-1α. Box plots are mean (±SEM) expression values of *jasmonate-responsive ETHYLENE RESPONSE FACTOR 4* (*JRE4*; A, C, E); and *glycoalkaloid metabolism 1* (*GAME1*; B, D, F) per treatment ($n = 5$) measured at the nematodes' invasion (A and B), galling (C and D) and reproduction (E and F) stages, respectively. Different lower-case letters above the box plots indicate significant differences in mean expression among treatments, determined via multiple comparisons Tukey's HSD test after ANOVA at $P \leq 0.05$.

*M. incognita* infection alone (Figs 2E–H, and 3C and D, yellow vs. blue box plots; **see Supporting Information—Table S5**). During the reproduction stage, *S. exigua* herbivory on *M. incognita*-infected plants significantly upregulated *LapA* (Fig. 2J, yellow box plot; **see Supporting Information—Table S5**), whereas it had no significant effect on the other marker genes compared to plants infected with *M. incognita* alone (Figs 2I, K and L, and 3E and F, yellow box plots; **see Supporting Information—Table S5**). By comparing the double-infected plants to control plants and those infected with *S. exigua* only, it became clear that the downregulation of *GluB* by *S. exigua* (Fig. 2L; **see Supporting Information—Table S5**) is not affected by *M. incognita* infection. On the other hand, the significant main effects of *M. incognita* on the expression of *JRE4* and *GAME1* at the galling and reproduction stages were not changed by *S. exigua* feeding (Fig. 3C and D, and E and F, blue and yellow box plots; **see Supporting Information—Table S5**). Therefore, our results collectively suggest that *S. exigua* can affect nematode-induced root responses, in particular via the JA-signalling pathway, depending on the nematodes' infection stage.

## Discussion

The goal of our study was to determine whether the impact of AG feeding on root defence responses induced by *M. incognita* depends on the nematodes' life cycle. We tested this by exposing *S. exigua* caterpillars to tomato plants infected by *M. incognita* at different stages of the root infection cycle. We found that *S. exigua* affected *M. incognita* root-induced responses, mainly at the nematodes' reproduction stage. In particular, the JA-signalling pathway was affected, as evidenced by lowered levels of JA in double-infected plants compared to plants infected with *M. incognita* alone. Jasmonic acid is known to regulate the GAME pathway in tomato via the *JRE4* transcription factor (Thagun *et al.* 2016). In this study, the attenuation of the JA pathway did neither lower α-tomatine concentrations nor the expression of the GAME genes (*JRE4* and *GAME1*) in double-infected plants compared to plants challenged with *M. incognita* alone (Fig. 4). This may be because the glycoalkaloid biosynthesis transcriptional coordinator *JRE4* can act downstream of JA signalling (Abdelkareem *et al.* 2017). Caterpillar feeding also enhanced *LapA* expression in double-infected plants at the nematodes' reproduction stage compared to plants challenged with *M. incognita* alone. *LapA* acts downstream of JA signalling as a modulator of late wound-induced responses (Fowler *et al.* 2009). *LapA* expression is induced by external application of ABA, methyl jasmonate (MeJA) and ET (Chao *et al.* 1999). Here, the levels of ABA in double-infected plants remained elevated, which could be related to the upregulation in *LapA* expression. Cross-talk between phytohormones is widely recognized as a mechanism to tailor herbivore-induced responses to specific combinations of attackers (Pieterse *et al.* 2009; Zamioudis and Pieterse 2012). Cross-talk between the JA-signalling pathway and both the SA and ABA pathways may also explain why glycoalkaloid levels remained increased in double-infected plants at the nematodes' reproduction stage compared to plants infected with *M. incognita* alone, despite lowered JA levels, *GluB* expression and no effect on *LoxD* expression compared to *M. incognita*-infected roots (Fig. 4). This cross-talk of SA and ABA with the JA pathway might also occur downstream of JA biosynthesis, e.g. at the level of transcription factors like MYC or ERF and in our case, *JRE4* (Fig. 4).

To date, the elicitation of root defences by endoparasitic nematode infection at later time points in their life cycle is virtually undescribed; most papers focus on signalling events occurring at 1–7 days after infection (Kyndt *et al.* 2012a, b; Kammerhofer *et al.* 2015; Martínez-Medina *et al.* 2017). Here, we found that *M. incognita* infection at the invasion and galling stages did not elicit strong defence responses, either on the level of phytohormones, gene expression or glycoalkaloid production. The lack of significant defence induction during the invasion stage can be partly attributed to how the RKNs migrate inside the roots. Once the J2s of RKN are inside roots, they avoid damaging plant cells by moving intercellularly through soft tissues of the host plant root tissues (Gheysen and Mitchum 2011; Gheysen and Jones 2013). Also, RKNs secrete effector proteins that play an essential role during both the penetration (invasion) and the establishment and galling phases. These effectors suppress host defence responses and help the nematode to establish a permanent feeding site (Abad and Williamson 2010; Mitchum *et al.* 2013). For instance, the rice pathogenic nematodes *M. graminicola* and *M. javanica* excrete the effectors, *Mg-MSP18* and *Mj-MSP18*, between 7 and 21 dpi to suppress the activation of their host's immune responses, such as the hypersensitive response (Grossi-de-Sa *et al.* 2019). In our study, *M. incognita* did not induce significant root defences at the galling stage. We correlate this lack of defence induction to the fact that *M. incognita* utilizes effector proteins to repress plant responses in roots during the galling stage. For example, when feeding on *A. thaliana*, *M. incognita* secretes the effector *Mi-CTR* into the roots. This lowers pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) by suppressing the transcription of defense genes, such as *WRKY33,29, PDF1.2* and *pathogen related protein-1 (PR1)* (Jaouannet *et al.* 2013). The effect of *Mi-CTR* occurs after root invasion and initiation of the giant cells 21 dpi most likely to ensure successful establishment (Jaouannet *et al.* 2013).

Interestingly, when *M. incognita* reached the reproduction stage, we observed an induction of defence responses. We found that *M. incognita* infection increased all phytohormone levels measured, as well as the concentration of α-tomatine and the expression of its biosynthesis gene *GAME1*. Possibly, the swelling of the female bodies with the ripening eggs intensifies the cell damage at the feeding sites, leading to the observed hormonal and defence responses. It is remarkable, however, that the expression patterns of defence-signalling marker genes are not affected in the same way. Possibly the expression of defence marker genes might be regulated by effector proteins that are only secreted by female RKN during the reproduction stage. For instance, the *Misp12* effector is specific to *M. incognita* and secreted by mature females at least 28 dpi (Xie *et al.* 2016). Overexpression of *Misp12* suppresses *PR1* and *phenylalanine ammonia-lyase-5 (PAL5)* genes (SA pathway markers) in *N. benthamiana*. In *Misp12*-silenced plants, an upregulation of the *proteinase inhibitor 2 (Pin2)* (JA pathway marker) is reported. The authors suggest that *Misp12* might be involved in the maintenance of giant cells during the reproduction stages (Xie *et al.* 2016).

The systemic effect of *S. exigua* feeding on root hormone levels and defence responses was much less pronounced than local nematode-induced responses. On the one hand, this may be because the caterpillars fed only for 24 h on the plant, while the nematodes were continuously feeding. In other studies, shoot feeding by herbivores, including *S. exigua* and *Pieris rapae*, was applied for 2–7 days before defence responses were observed in the roots (Danner *et al.* 2015; Kafle *et al.* 2017). Possibly, 24 h of AG feeding may have been too short to elicit strong systemic responses in tomato roots. Moreover, systemic responses are generally weaker than locally induced responses (van Dam *et al.* 2001; Babst *et al.* 2009; Ádám *et al.* 2018). For example, leaf feeding by diamondback moth caterpillars in *Brassica oleracea* elicited slight systemic JA responses in the roots compared to the local induction by *Delia radicum* (Karssemeijer *et al.* 2020). In another study, shoot feeding by *P. rapae* larvae on *B. rapa* plants elicits much lower root volatile emissions than local damage by *D. radicum* larvae (Danner *et al.* 2015).

Interestingly, we found that the age of the plant affects the systemic response as well. In our experimental set-up, we applied nematode eggs at one single time point. Consequently, the *S. exigua* caterpillars were placed on tomato plants that were at different ages and ontogenetic stages. The expression of some defence marker genes was significantly upregulated (Fig. 2I) or downregulated (Fig. 3F) by *S. exigua* feeding only in the last batch of plants, which were 8 weeks old and flowering. It has been reported that herbivore-induced plant responses can significantly change as a function of plant ontogenetic stage (Quintero and Bowers 2011, 2012). For instance, the concentration of iridoid glycosides in *Plantago lanceolata* roots after AG herbivory was twice as high in mature plants compared to young plants (Quintero and Bowers 2011).

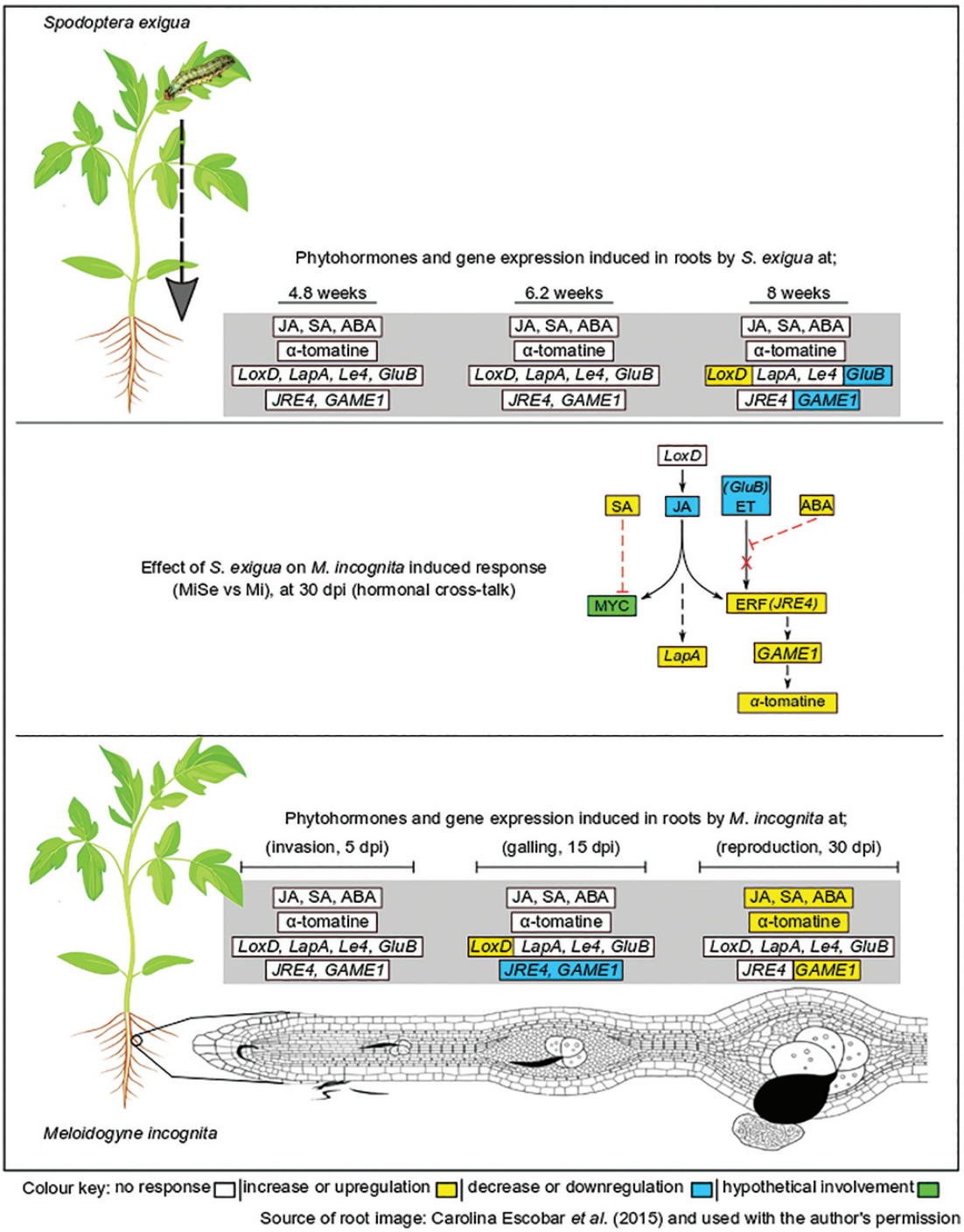


**Figure 4.** Interactions between root defence responses upon root and leaf herbivory. Schematic illustration of induced defences in tomato roots including the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), the glycoalkaloid α-tomatine and defence genes (*Lipoxygenase D* (*LoxD*), *Leucine aminopeptidase A* (*LapA*), *Le4 abscisic acid-responsive*, *Basic-β-1-glucanase* (*GluB*)) and glycoalkaloid metabolism (GAME) genes (*jasmonate-responsive ETHYLENE RESPONSE FACTOR 4 transcription factor* (*JRE4*) and *GLYCOALKLOID METABOLISM 1* (*GAME1*)). The top panel represents phytohormones and gene expression induced in tomato roots by the caterpillar *Spodoptera exigua* on plants of different ages (4.8, 6.2 and 8 weeks). The bottom panel represents phytohormones and gene expression induced in tomato roots by the root-knot nematode (RKN) *Meloidogyne incognita* at different root infection cycle stages (invasion stage estimated at 5 days post-nematode inoculation (dpi), galling stage estimated at 15 dpi and reproduction stage estimated at 30 dpi). The middle panel shows the effect of *S. exigua* leaf feeding on root responses induced by *M. incognita* (MiSe) compared to those infected with *M. incognita* (Mi) alone at 30 dpi (hormonal cross-talk). White boxes: no response, yellow boxes: increase in trait levels or upregulation of gene expression, blue boxes: decrease in traits levels or downregulation of gene expression and green box: hypothetical involvement. In the proposed hormonal cross-talk schedule in the middle, dotted red lines show negative cross-talk, the black arrows show the steps in the JA pathway and the dashed black arrows represents several unknown steps. In our cross-talk model, we propose that the increase in SA affects the JA pathway negatively at the level of the MYC transcription factor. At the same time, the increase in ABA levels blocks the ethylene (ET) pathway, which regulates the ETHYLENE RESPONSIVE FACTOR (ERF) branch of the JA pathway. We hypothesize that the absence of ET promotes the activity of the *JRE4* transcription factor, which enhances transcription of the GAME pathway. Based on the response of the defence marker gene *LapA* in MiSe plants at 30 dpi, we also hypothesize that this pathway leading to late JA responses is involved in the interaction.

In nature, plants are likely to interact with AG herbivores and RKN at the same time. Here we found that *S. exigua* herbivory differentially affects the root-induced responses by *M. incognita* in tomato roots. These effects occurred in dependence on the life cycle of the nematode, whereby the impact was the strongest in the reproductive stages. Herbivore identity and sequence of arrival on the target host plant are some of the critical factors shaping interactions between AG–BG herbivores (Erb *et al.* 2011; Sarmento *et al.* 2011; Kafle *et al.* 2017). We conducted our experiment by first infecting the plants with RKN. This is likely the natural sequence of arrival because the roots develop before the shoots after seed germination. Moreover, nematodes are ubiquitous in natural systems. Roots are therefore likely to be invaded with nematodes before herbivores arrive on AG organs (Hoysted *et al.* 2018; van Dam *et al.* 2018). *Spodoptera exigua* feeding on *M. incognita*-infected plants reduced JA but not SA concentrations. In a similar study, *M. incognita* were allowed to colonize tomato plants that had experienced 7 days of *S. exigua* feeding, followed by a lag phase of another 7 days (Kafle *et al.* 2017). The authors found that after 14 days of *M. incognita* infection, the root JA levels decreased in tomato plants that were previously damaged by *S. exigua*. Combining our results with this study, we conclude that it may not matter whether the nematode or the AG herbivore infects first; AG feeding seems always to reduce RKN-induced JA levels in the roots.

Jasmonates are essential regulators of systemic signalling between AG and BG tissues (Wasternack 2007; Wasternack and Hause 2013). It has been established that JAs regulate the steroidal glycoalkaloid metabolism pathway via the *JRE4* transcription factor (De Geyter *et al.* 2012; Cárdenas *et al.* 2016; Thagun *et al.* 2016). Here the expression of *JRE4* was not altered by *S. exigua* feeding alone, nor did the caterpillar alter the *M. incognita*-induced upregulation of this transcription factor. Notably, the expression of *LapA* (JA marker) was significantly upregulated in double-infected plants compared to plants infected with *M. incognita* only, while *LoxD* expression was similar when *S. exigua* co-occurred with *M. incognita*. Our results suggest that the interaction between *M. incognita* and *S. exigua* might rely on the induction of late wounding responses regulated by *LapA* downstream of JA synthesis, e.g., on transcription factor level (Fig. 4). Unfortunately, our experimental set-up did not allow us to precisely determine the role of *LapA* because the plants with RKN in different life cycle stages also differed in age. *LapA* might also be associated with plant development, especially in the flowering stage, as reported by Chao *et al.* (1999).

Finally, the induction of JA levels by *M. incognita* infection was accompanied by an increase in α-tomatine production. Increases in JA and α-tomatine concentrations upon nematode attack or exogenous application of elicitors, such as MeJA, have been reported in tomato and other plant species (Abdelkareem *et al.* 2017; Kafle *et al.* 2017). Glycoalkaloids are usually associated with increased generalist herbivore resistance (Ökmen *et al.* 2013; Abdelkareem *et al.* 2017). In our study, we did not measure the ecological consequences associated with these defence responses, e.g., for later arriving herbivores. Further studies to test the effects of α-tomatine on the performance of the RKNs may reveal their effectiveness as defences against this generalist herbivore.

## Conclusions

Our study examined the impact of AG chewing herbivores on root-induced responses by RKN at different life cycle stages. We found that both the AG chewing herbivore and the RKN affect root defences. The effect of root infection by RKN alone, as well as the effect of AG herbivory on RKN-induced root defence responses, depends on the nematode's life cycle stage. Studies testing the impact of long periods of AG herbivory on nematode-induced root responses are needed to reveal how the interactions with BG responses might change over longer interaction times. Such studies will help to optimize tomato breeding efforts towards cultivars with high resistance to AG and BG insect pests and pathogens.

## Supporting Information

The following additional information is available in the online version of this article—

**Figure S1**. The number of root galls counted in tomato plants roots upon root infection by *Meloidogyne incognita*.

**Table S1**. List of primers sequences used for quantitative polymerase chain reaction (qPCR).

**Table S2**. Permutational multivariate analysis of variance (PERMANOVA) results based on Gower dissimilarities on phytohormone and α-tomatine data for herbivory effects of *Meloidogyne incognita* root-knot nematodes (RKNs) and the caterpillars of *Spodoptera exigua*.

**Table S3**. Two-way factorial analysis of variance (ANOVA) results on phytohormone and α-tomatine for the herbivory effects of *Meloidogyne incognita* root-knot nematode (RKN) and the caterpillars of *Spodoptera exigua*.

**Table S4**. Permutational multivariate analysis of variance (PERMANOVA) results based on Gower dissimilarities on gene expression data for herbivory effects of *Meloidogyne incognita* root-knot nematode (RKN) and the caterpillars of *Spodoptera exigua*.

**Table S5**. Two-way factorial analysis of variance (ANOVA) results on gene expression for the herbivory effect of *Meloidogyne incognita* root-knot nematode (RKN) and the caterpillars *of Spodoptera exigua*.

## Data Availability

The data underlying this study are published as open access at the iDiv Data Repository (Mbaluto *et al.* 2020; http://idata.idiv.de/ddm/Data/ShowData/1816).

## Sources of Funding

All authors gratefully acknowledge the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118). C.M.M. acknowledges the Deutscher Akademischer Austauschdienst (DAAD) for a doctoral research grant (research grant no: 91607343). E.M.A. acknowledges the DAAD-German Egyptian Research Short-Term Scholarship Program (GERSS) scholarship awarded to conduct part of her Master of Science Research at iDiv. M.F. acknowledges the Go Global Self-Directed Research Award of the University of British Columbia for support to conduct research training at the iDiv facilities.

## Acknowledgements

All authors thank Dr. Adriaan Verhage of Rijk-Zwaan, The Netherlands, for providing the root-knot nematode eggs, Dr. Katharina Grosser, Dr. Fredd Vergara and Anne Maedicke for

their technical support in molecular and chemical analysis, Dr. Jitendra Gaikwad and the iDiv Data repository team for support in data preparation, quality check and uploading of the data to the iDiv repository. Lastly, all authors thank the two anonymous reviewers and the associate editor for their helpful and constructive comments on the earlier versions of the manuscript.

## Contributions by the Authors

C.M.M., A.M.M. and N.M.v.D.: conception of the idea and experimental design; C.M.M.: execution, processing of samples, data analysis; E.M.A. and M.F.: processing of samples, data analysis; C.M.M.: writing of the initial manuscript; C.M.M.: deposition of data in iDiv data repository; A.M.M. and N.M.v.D.: critical revision of draft manuscripts and approval of the final manuscript for submission.

## Conflict of Interest

The authors declare that this research article was conceptualized, designed and drafted in the absence of any commercial interest or financial obligations that could be construed as a potential conflict of interest.

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# CHAPTER 4

# Induced local and systemic defense responses in tomato underlying interactions between the root-knot nematode *Meloidogyne incognita* and the potato aphid *Macrosiphum euphorbiae*

# Induced local and systemic defense responses in tomato underlying interactions between the root-knot nematode *Meloidogyne incognita* and the potato aphid *Macrosiphum euphorbiae*

Crispus M. Mbaluto[1,2], Nicole M. van Dam[1,2], Ainhoa Martinez-Medina[1,3]

[1]Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig; Leipzig, Germany, Deutscher Platz 5e, 04103 Leipzig Germany; [2]Institute of Biodiversity, Friedrich-Schiller-Universität-Jena; Jena, Germany, Dornburger Str. 159, 07743 Jena Germany; [3]Plant-Microorganism Interaction Unit, Institute of Natural Resources and Agrobiology of Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008, Salamanca, Spain

**Manuscript in preparation**

## ABSTRACT

Plants can be attacked belowground (BG) and aboveground (AG) by different pathogens and herbivores. Locally and systemically induced defense responses mediate these AG-BG interactions. Here, we investigated how root infection by the root-knot nematode (RKN) *Meloidogyne incognita* affects the AG defense responses triggered by the potato aphid *Macrospiphum euphorbiae* as well as the impact aphid feeding has on root responses triggered by the RKN in tomato plants. As plant defense responses to RKN vary over their life cycle, we investigated the interaction between RKN and aphids at different stages of the nematodes' infection cycle, *i.e.,* at the invasion, galling, and reproduction stages. We analyzed leaf and root levels of the phytohormones jasmonic acid (JA), JA-isoleucine (JA-Ile), salicylic acid (SA), abscisic acid (ABA), and auxin (indole-3 acetic acid: IAA), as well as the expression dynamics of marker genes in the JA (*proteinase inhibitor II*, *PI-II*) and SA (*pathogen-related protein 1*, *PR1*) pathways. Also, we measured the levels of the steroidal glycoalkaloids (SGAs) *α*-dehydrotomatine and *α*-tomatine and the expression of glycoalkaloid metabolism (GAME) genes; *jasmonate-responsive ETHYLENE RESPONSE FACTOR (ERF) transcriptional factor 4* (*JRE4*) and glycoalkaloid metabolism 1 (*GAME1*). Our results showed that aphid feeding neither affected the levels of phytohormones nor the expression of *PI-II* and *PR1* in leaves. However, SGAs levels and the expression of GAME genes decreased in leaves in 4.5-6 weeks old plants infested with aphids only. In the roots, aphid feeding decreased the levels of JA, ABA, and IAA, but only in plants that were 8 weeks old. Aphid's feeding neither changed SGAs levels nor the expression of GAME genes systemically in roots. Nematode root infection increased root SA levels throughout the infection cycle and ABA levels only at the reproduction stage. Levels of SGAs and the expression of GAMEs increased locally at the galling stage. Leaf SA levels increased in plants where RKNs were at the reproduction stage. SGA levels and the expression of GAME genes decreased in leaves of plants with RKNs at the invasion stage. Aphid feeding on nematode-infected plants did not alter the systemic effects of nematodes on SA showed only mild effects on the leaf phytohormone and SGA levels, and the GAME pathway. In roots of

double infested plants, root JA-Ile levels were higher when RKNs were in the galling stage. In the same plants, *PI II* expression, a marker for the JA pathway, was lower in roots. These differences did not translate in differences in GAME expression or SGAs in roots between plants with only RKN in the galling stage and double infested plants. In none of the other developmental stages, there was an interactive effect of aphid feeding on nematode-induced roots responses. This means that, overall, nematode feeding had a stronger effect on AG aphid-induced responses, than the reverse. The stage of the RKN nematode co-determined the strength of the effect.

**Keywords:** above-belowground interaction, endogenous phytohormones, gene expression, life cycle stages, steroidal glycoalkaloid metabolism, plant-mediated interactions, potato aphid (*Macrosiphum euphorbiae*), root-knot nematodes (*Meloidogyne incognita*), steroidal glycoalkaloids

## INTRODUCTION

Plants are often attacked by several species of insect herbivores and pathogens that reduce their fitness. To defend themselves against the attackers, plants have evolved multifaceted mechanisms to perceive and appropriately respond to the specific attackers, thus preventing or attenuating the attack (Mithöfer and Boland, 2008; War et al., 2012; Mortensen, 2013). Plant hormones regulate the plant's immune system (Pieterse et al., 2012). Among them, jasmonic acid (JA) with its derivates (collectively known as jasmonates JAs) and salicylic acid (SA) are considered as major defense hormones (Pieterse et al., 2009, 2012; Erb et al., 2012). The activation of these phytohormone pathways occurs with considerable specificity. The JA responsive pathway is typically (but not exclusively) activated upon the attack of chewing herbivores and necrotrophic pathogens, while piercing-sucking herbivores and biotrophs trigger the SA responsive pathway (Walling, 2000; Zhu-Salzman et al., 2004; Howe and Jander, 2008; Diezel et al., 2009; Lemarié et al., 2015; Wasternack, 2015). While the JA and SA pathways form the backbone of the plant's immune system, other hormones such as ethylene, abscisic acid (ABA), auxins and cytokinins also contribute to defense signaling (Bari and Jones, 2009; Erb et al., 2012; Kammerhofer et al., 2015a). These hormones can antagonistically or synergistically interact with the SA-JA backbone of the plant's immune signaling network. This so-called hormone cross-talk provides the plant with a powerful capacity to finely regulate its immune response to the specific attacker (Pieterse et al., 2009; Li et al., 2019).

The induction of plant defense responses upon herbivory at local sites is often accompanied by systemic induced responses in distal tissues, thereby protecting undamaged plant parts from subsequent attack. Systemic signaling is not limited to the particular compartment (roots or shoots) under attack, but it can cross the root-shoot interface. Several studies show that the attack by aboveground (AG) and belowground (BG) herbivores and pathogens lead to systemic responses mediated via the plants to influence organisms on the opposite compartment. Indeed, BG herbivores can induce systemic responses in AG plant parts that can facilitate or impede the performance of the AG insect herbivores. For example, root damage

by the insect herbivore *Tecia solanivora* and the parasitic root nematode *Meloidogyne incognita* decrease the performance of the AG insect herbivores *Spodoptera exigua*, *Spodoptera friguperda* and *Tuta absoluta* in potato and tomato (Kumar et al., 2016; Arce et al., 2017). By contrast, the shoot-feeding aphids *Myzus persicae* and *Brevicoryne brassicae* preferred feeding on plants infected in roots by the parasitic root nematodes *Globodera pallida* and *Meloidogyne hapla* compared to non-infected plants (Hoysted et al., 2017; van Dam et al., 2018). Although less studied, AG herbivory can also systemically influence the performance of herbivores feeding on the BG plant parts. For example, AG herbivory by *Manduca sexta* on *Nicotiana attenuata* enhances the performance of the parasitic root nematode *M. incognita* (Machado et al., 2018). In contrast, AG herbivory can also negatively affect BG feeding herbivores. For example, leaf-feeding by *Spodoptera littoralis* on maize plants deterred larvae of *Diabortica virgifera* from infesting the roots (Erb et al., 2015). In another study, leaf damage by *Pieris brassicae* on *Brassica nigra* decrease the performance of the root herbivore *Delia radicum* by 50 % (Soler et al., 2007).

These AG-BG interactions likely are because herbivory triggers pathways that are responsible for the production of toxic and deterrent compounds, as well as changes in primary metabolites at both local and systemic sites. These changes in the plant's primary and secondary chemistry may underpin, at least partially, the observed effects on the interactors. For instance, root herbivory and elicitation by the application of JA results in the accumulation of secondary metabolites, including steroidal glycoalkaloids, glucosinolates, and nicotine, in shoots of different plant species such as *Solanum* spp. and *Brassicaceae* (Fragoso et al., 2014; Kumar et al., 2016; Bakhtiari et al., 2018). On the other hand, shoot herbivory or elicitation by exogenous application of methyl jasmonate (MeJA) leads to the accumulation of phytohormones, steroidal glycoalkaloids, phenolic acids, glucosinolates in roots of, amongst others, *Solanum* spp., *Zea* spp., *Brassica* spp. (Hlywka et al., 1994; Soler et al., 2007; Abdelkareem et al., 2017; Chapter 3 of this thesis). Such systemically-triggered changes in plant secondary chemistry can affect the performance of herbivores feeding on the "other" compartment (Kumar et al., 2016; Bakhtiari et al., 2018; van Dam et al., 2018).

Even though the number of studies investigating the mechanisms driving plant-mediated AG-BG interactions is rapidly rising, the data are still fragmented. This makes it a challenging effort to draw general conclusions on these mechanisms. It has been suggested that the variability in the outcome of induced responses depend on the system under investigation and the combination of herbivores for each study. As an example, root infection by the cyst nematode *Heterodera glycines* sometime reduces the performance of the aphid *Aphis glycine*, but in other cases, the effects are inconsistent (Hong et al., 2010; McCarville et al., 2012). Interestingly, it has been demonstrated that even the same induced defense responses can differentially affect the herbivores. For example, root herbivory on potato, maize, tobacco, and other plants triggers the production of chlorogenic acid in leaves after insect herbivory. Some of these studies report

that chlorogenic acid negatively affects the AG insect herbivores, while others show no effect (Johnson and Felton, 2001; Beninger et al., 2004; Erb et al., 2009; Kumar et al., 2016).

The majority of the abovementioned studies on AG-BG plant-mediated interactions focus on insect herbivores. Interactions involving plant-parasitic root nematodes such as the root-knot nematodes (RKNs) and AG feeding aphids remain less investigated. RKNs are soil-inhabiting parasites and infect the roots of nearly all higher plant species. These nematodes, either alone or in combination with other pathogens, constitute agro-economically important crop pests. Overall, their effects in natural and agroecosystems are estimated globally to cause approximately 14 % losses of life-sustaining and economically important crops and plants (Abad et al., 2008; Jones et al., 2013; Bernard et al., 2017; Ibrahim et al., 2019). They are obligate root feeders, which spend most of their life-cycle inside roots, thereby influencing root physiology throughout their life cycle. When the eggs hatch, the infective stage two juveniles (J2s) migrate in the soil, penetrate their host plant roots near the zone of elongation, and migrate intercellularly to reach the root tip. They then turn around past the Casparian strip to enter into the vascular cylinder (Fenoll et al., 1997; Perry et al., 2009). They select several vascular phloem cells to induce their feeding sites, called the giant cells. As they feed and develop further, they secrete and inject effector molecules that cause hyperplasia and hypertrophy of cells surrounding the giant cells to form root galls. The J2s molt twice to J3 and J4 stages to reach the adult stage, after which the body of females swells up and becomes pear-shaped. The females produce eggs that are deposited on the root cortex. Overall, their life cycle can be divided into three major stages: root invasion, feeding site development, and reproduction stages (Chapter 3 of this thesis). It has been shown that plant responses mounted against RKN infection differ according to the specific stages of the infection. For example, at the early stages of root infection, RKNs trigger the activation of phytohormonal signaling pathways as well as secondary metabolic pathways in tomato (Brenner et al., 1998; Martínez-Medina et al., 2017). During its development, the RKN affects the expression of genes with significant homology to peroxidases, and trypsin protein inhibitors occur during the formation of feeding sites and in young galls in *Arabidopsis thaliana* (Vercauteren et al., 2001; Jammes et al., 2005). At mature developmental stages, the RKN induces phytohormones, steroidal glycoalkaloids, and primary metabolites in roots of tomato (Afifah et al., 2019; Chapter 3 of this thesis). Besides the modulation of defense responses in roots, several studies demonstrate that root infection by RKNs also alter defense-related compounds in AG plant organs. However, the studies dealing with the impact of RKN on AG defenses responses are scarce and show conflicting results. Indeed, a study by Hamamouch et al. (2011), shows that root infection by the RKN *M. incognita* sometimes upregulates the expression of some marker genes for the JA and SA pathways, whereas in other cases they are suppressed in leaves.

Aphids are phloem sucking insect herbivores that are also economically important pests. High population numbers result in heavy siphoning of phloem sap, which can devastate the host plant and thus

reduce yields. They also transmit disease-causing pathogens that additionally reduce the plant's performance (Baranyovits, 1973; Guerrieri and Digilio, 2008). In analogy to RKNs, aphids feed on vascular contents. They insert their mouthparts (stylet) in between the primary and secondary cells layers to reach the sieve elements in the vascular tissues. Large radial intercellular spaces between parenchyma cells facilitate the smooth movement of the stylet (Will and van Bel, 2006; Guerrieri and Digilio, 2008). Aphids are 'stealthy' feeders because they cause minimal damage to the host plant compared to other feeding guilds, e.g., chewers (Voelckel et al., 2004). Plants generally respond to aphid attack by activating the SA responsive pathway (Walling, 2000; De Vos et al., 2005), although some studies revealed the activation of the JA pathway upon aphids attack (Fidantsef et al., 1999; Cooper and Goggin, 2005). In order to counteract the plant's defense responses, aphids inject effector proteins that interfere with the defense signaling of the host plant (Will et al., 2007; Walling, 2008; Hogenhout and Bos, 2011; Kettles and Kaloshian, 2016). Remarkably, it has been shown that aphids can trigger systemic induction of defenses in roots and influence BG biota. For example, root exudates secreted by potato plants infested with the green peach aphid (*M. persicae*) promoted the recruitment of the Rhizobacteria *Paenibacillus polymyxa E68* in pepper (*Capsicum annuum* L. 'PR') plants (Kim et al., 2016) and also impaired the hatching of the cyst nematode *Globodera pallida* cysts in soil (Hoysted et al., 2018). Shoot herbivory by the aphid *Brevicornye brassicae* triggered an increase in JA levels in *Brassica oleracea,* but this did not impair the development of root fly *D. radicum* (Karssemeijer et al., 2020).

In this study, we aimed to disentangle the molecular and chemical mechanisms driving the plant-mediated interaction between parasitic root nematodes and aphids. By establishing a system including the important crop species tomato (*Solanum lycopersicum*), we addressed the effects of root infection by the RKN *M. incognita* on shoot defenses induced by the aphid *Macrosiphum euphorbiae*, as well as and the impact of leaf herbivory by the aphid *Ma. euphorbiae* on root defenses triggered by the RKN *M. incognita*. Because interactions between AG and BG induced plant responses depend on the RKN infection stage (Chapter 3 of this thesis), we infested plants with the aphid *Ma. euphorbiae* at particular stages – invasion, galling, or reproduction - of the RKN *M. incognita*. The results obtained showed that RKN had a mild effect on defense responses triggered by the aphid. On the other hand, the aphid did not interfere with any of the RKN-induced root defense responses. This indicates that the RKN induces a strong effect that can not be overruled by the aphid, a phenomenon called canalization.

## MATERIALS AND METHODS

### Nematode and aphid cultures

We used the RKN *M. incognita* as the BG herbivore and the potato aphid *Ma. euphorbiae* as the AG herbivore. The RKN colony was initially sourced from Rijk Zwaan (De Lier, The Netherlands) and

maintained on tomato cv 'Moneymaker' in the glasshouse. The colony was initiated from a single egg mass, and eight weeks later, eggs were extracted for use in the experiments (Martínez-Medina et al., 2017). The potato aphid was kindly provided by Dr. Zeger van Herwijnen (Rijk Zwaan Breeding B.V De Lier, The Netherlands). We maintained a laboratory colony using the leaf disc method (Rocca and Messelink, 2017) with slight modifications. In brief, we prepared 1 % (w/v) water-agar and poured in plastic boxes 8 cm (length) x 5 cm (width) x 4 cm (height) to obtain approximately 0.5 cm thickness. A leaf disc from *Capsicum annuum* was embedded on the solidified agar with the abaxial side facing up to mimic normal aphid feeding side or position. The colony was maintained in a growth chamber (CLF PlantClimatic, CLF PlantClimatics GmbH, Wertingen, Germany) under 12-h light, 22 °C : 12-h dark, 20 °C , 45 % relative humidity conditions. In the bioassays, we used apterous individuals.

**Plant material and growth conditions**

We used tomato (*S. lycopersicum*) cultivar 'Moneymaker', as the model plant. Tomato seeds were obtained from Intratuin B.V (Woerden, the Netherlands). The seeds were sterilized, germinated, and transplanted, according to Chapter 3 of this thesis. In the glasshouse, the plants were randomly distributed and grown for three weeks under 16-h light 25 ±3 °C: 8-h dark 22 ±3, 40 % relative humidity conditions. The plants were watered as required and supplemented with half-strength Hoagland solution weekly (Hoagland and Arnon, 1938). Three weeks after transplanting, we used the plants in the bioassays.

**Nematode inoculation and aphid infestation**

We used a complete randomized block design, with factors (1) root challenged with the RKN *M. incognita* and (2) leaf challenged with the aphid *Ma. euphorbiae*. The plants assigned for *M. incognita* inoculation received 3000 *M. incognita* eggs suspended in 1ml of tap water, according to Chapter 3 of this thesis. Plants not assigned for nematode inoculation were mock-inoculated with 1 ml of tap water. We established three-time points after the nematode inoculation, corresponding to the main stages of the RKN *M. incognita* life cycle stages: 5 days after nematode inoculation, corresponding to the invasion stage; 15 days corresponding to the galling stage, and 30 days corresponding with the reproduction stage (Chapter 3 of this thesis). At each specific time point after nematode inoculation, plants assigned to the AG herbivore were challenged with 12 *Ma. euphorbiae* individuals of mixed-ages. The aphids were contained on a single leaf for 24 hours, using a round clip cage of 7 cm in diameter. The clip cage was mounted on one fully expanded leaf close to the tip (Bandoly and Steppuhn, 2016; Chapter 3 of this thesis). Similarly, we mounted an empty clip cage on the plants not assigned for the aphid infestation. At each specific time point after nematode inoculation, we established the following treatments: (1) controls: plants not challenged with any of the herbivores, (2) plants root-infected with the RKN *M. incognita*, (3) plants exposed to leaf-feeding by the aphid *Ma.*

*euphorbiae*, and (4) plants root-infected with the RKN *M. incognita* and exposed to the aphid *Ma. euphorbiae* on the leaves. Ten biological replicates of each treatment per time-point were established. At 24 hours after infesting the plants with aphids, we harvested the plants, starting with the leaves and followed with the roots samples. Leaves and roots materials were stored at -80 °C until use. Moreover, after washing the root systems, we counted and recorded the number of visible root galls from the RKN *M. incognita* infected plants (Table S1). In another experiment, we assessed the performance of the aphid on RKN infected plants by counting and comparing the number of nymphs produced in controls and. RKN infected plants (Table S2)

**Phytohormone extraction and analysis**

We extracted and quantified phytohormones from leaves and roots following the protocol previously described in Chapter 3 of this thesis, but with a modification for the nebulization step. The compounds were nebulized by electron spray ionization in the negative mode using the following conditions: capillary voltage 4500 eV, cone gas 35 arbitrary units /350 °C, probe gas 60 arbitrary units /300 °C and nebulizing gas at 60 arbitrary units. Data acquisition and processing were performed using the 'MS data Review' software (Bruker MS Workstation, version 8.2, Bruker, Bremen, Germany). Phytohormone levels were calculated based on the peak area of the corresponding internal standard and the amount of fresh mass of plant material (ng−1 mg−1 FW).

**Real-time quantitative RT-PCR**

Total RNA was extracted from ~100 mg (fresh weight) roots and leaves materials, according to Oñate-Sánchez and Vicente-Carbajosa (2008). We synthesized the first-strand cDNA from 1 µg DNase free RNA by reverse transcription using Revert Aid H-minus RT (Thermo Fisher Scientific Baltic UAB, Vilnius, Lithuania) following the manufacturer instructions. Real-time quantitative qPCR reactions and relative quantification of specific *m*RNA levels were performed according to Chapter 3 of this thesis, and gene-specific primers described in Table S3. The gene expression levels were determined by normalizing the data to a housekeeping gene *SIEF* (X14449), which encodes for the tomato elongation factor 1α (Miranda et al., 2013; Martínez-Medina et al., 2017). Normalized gene expression data were analyzed by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

**Extraction of steroidal glycoalkaloids and data processing**

We extracted ~100 mg fresh roots and leaves tissues following the method described in Chapter 3 of this thesis and with formic acid in methanol (0.05 % v/v) as solvent B of the mobile phase. Metabolite separation, characterization, and data processing were done according to Chapter 3 of this thesis. We

generated two datasets (i.e., roots and leaves) from which we selected all features with mass to charge ratio (*m/z*) of 578.4, 576.3, 416.3, and 414.3 at retention time 11-12 mins. These *m*/*z* values represent fragments of the tomatine and its aglycones (Cataldi et al., 2005). This was done for each study time point.

**Statistical analysis**

Datasets were analyzed using R software v 3.6.1 (R Core Development Team, 2019) unless indicated otherwise. For the phytohormones, steroidal glycoalkaloids, and the gene expression datasets, we used Two-way ANOVA linear models with *M. incognita* (Mi), *Ma. euphorbiae* (Me), and their interaction (Mi*Me) as model explanatory factors. Before the ANOVA analysis, all data sets were pre-screened for outliers using the interquartile range (IQR) method as a function in R. In cases where the ANOVA results were significant we detected the differences between the treatment groups using Tukeys Honest Significant Difference (HSD) for multiple comparisons at $P \leq 0.05$.

## RESULTS

### Impact of *Meloidogyne incognita* root infection on aboveground hormonal responses triggered by *Macrosiphum euphorbiae*

We started by analyzing the local effect of the aphid *Ma. euphorbiae* on leaf hormonal responses when feeding on plants alone. Because we used the nematodes' infection cycle stages to time the experiment, control plants and plants that were infested with aphids only, had different ages over the course of the experiment (invasion=4.5 weeks-old plants (young), galling =5.8 weeks-old plants (medium), reproduction=8 weeks-old plants (old)). We measured the levels of JA-Ile, SA, ABA, and IAA (JA levels were below the detection limit) (Figure 1), as well as the expression of the JA responsive gene *PI II* and the SA responsive gene *PR1* (Figure 2). We found that aphid feeding did not alter the concentrations of JA-Ile, SA, and ABA compared to the control plants, regardless of plant age (Figure 1A-C,E-G,I-K, green vs. purple boxplots, Table S4). In agreement with the hormonal responses, *Ma. euphorbiae* feeding did not affect the expression of *PI II* and *PR1* compared to controls (Figure 2, green vs. purple boxplots, Table S5). Remarkably, *Ma. euphorbiae* feeding on 8 weeks-old plants overall increased IAA levels (main effect; Table S4, $p$=0.0243), but there was no significant difference with control plants (Figure 1L, green vs. purple boxplot).

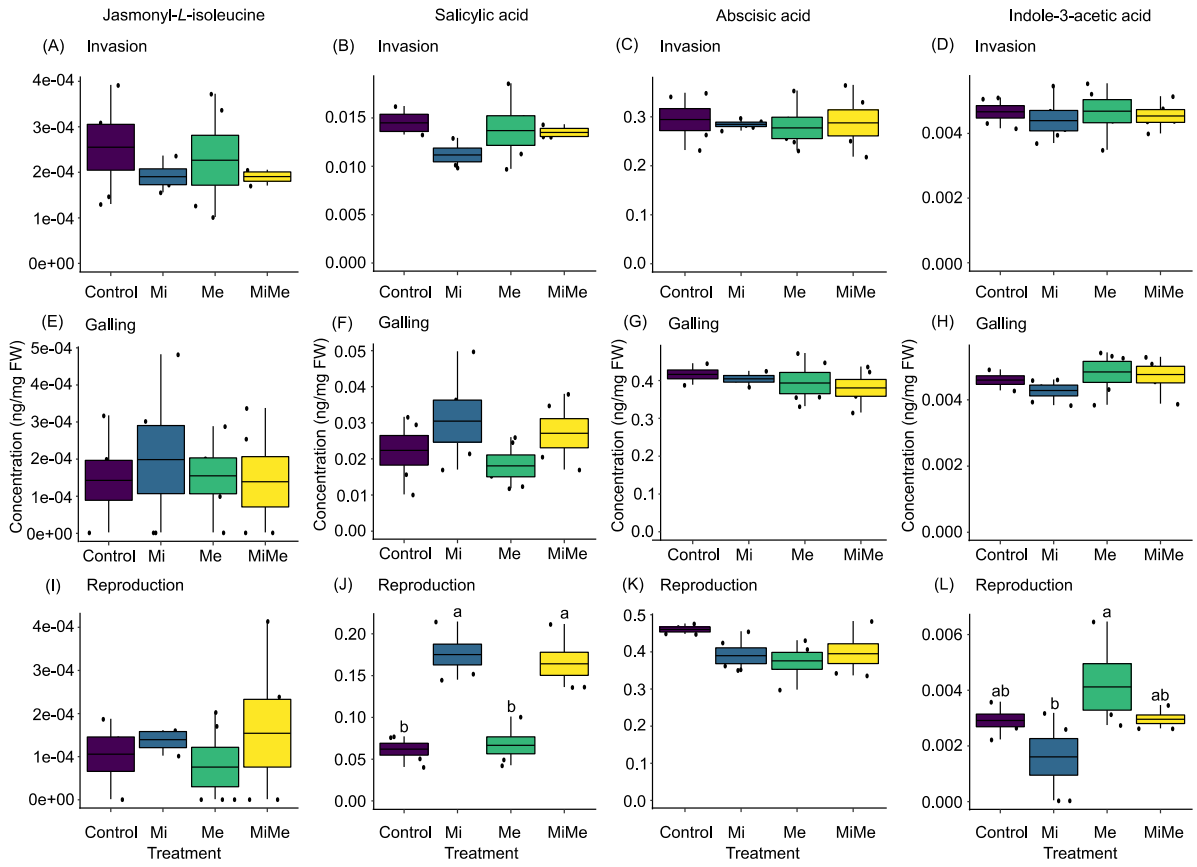


**Figure 1 Phytohormones concentrations in tomato leaves upon aboveground and belowground herbivory**. Mean concentrations (ng/mg fresh weight) of phytohormones in leaves of tomato plants infected belowground with *Meloidogyne incognita* (Mi), or infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Boxplots indicate the mean (±SEM, *n*=5) concentrations of abscisic acid (A,E,I), jasmonyl-*L*-isoleucine (B,F,J); salicylic acid (C,G,K); Indole-3- acetic acid (D,H,L) measured at the nematodes' invasion (A,B,C,D), galling (E,F,G,H) or reproduction (I,J,K,L) stages. Different letters above the boxplots indicate significant differences ($P \leq 0.05$) in mean values between treatments, determined by Tukeys HSD test after ANOVA.

Next, we studied the systemic impact of the RKN *M. incognita* root infection, throughout its infection cycle, on the leaf hormonal-related response. Nematode infection did not significantly affect the concentrations of JA-Ile, ABA, or IAA in tomato leaves compared to control plants at either of its infection cycle stages (Figure 1A,C,D,E,G,H,I,K,L, blue vs. purple boxplots, Table S4). In agreement with the JA-Ile levels, *M. incognita* infection did not affect the expression of the JA responsive gene *PI II* compared to controls, regardless of the infection stage (Figure 2A,C,E, blue vs. purple boxplots). Root infection by *M. incognita* significantly increased SA levels compared to controls, but only at the reproduction stage (Figure 1J, blue vs. purple boxplot, Table S4). In contrast, the expression of the SA-marker gene *PR1* in the *M.*

*incognita* infected plants was not significantly different from that in controls (Figure 2B,D,F, blue vs. purple boxplots, Table S5).

To decipher the systemic effect of RKN infection on AG phytohormonal-related responses triggered by the aphid *Ma. euphorbiae*, we compared plants challenged by both *M. incognita* and *Ma. euphorbiae* to those challenged with *Ma. euphorbiae* alone at each nematode infection cycle stage (Figures 1,2, yellow vs. green boxplots, Tables S4,S5). The concentrations of JA-Ile, ABA, and IAA, as well as the expression levels of *PI II* and *PR1* in leaves of plants challenged with both herbivores were not significantly different from those infested with the aphid *Ma. euphorbiae* alone (Figure 1A-I,K,L, and Figure 2, yellow vs. green boxplots, Tables S4, S5). The SA levels were similar in *Ma. euphorbiae* and double infected plants at the nematodes' invasion and galling stages. However, at the RKNs' reproduction stage, SA levels in double infected plants were increased compared to plants infested with *Ma. euphorbiae* alone (Figure 1B,F,J, yellow vs. green boxplots, Table S4).

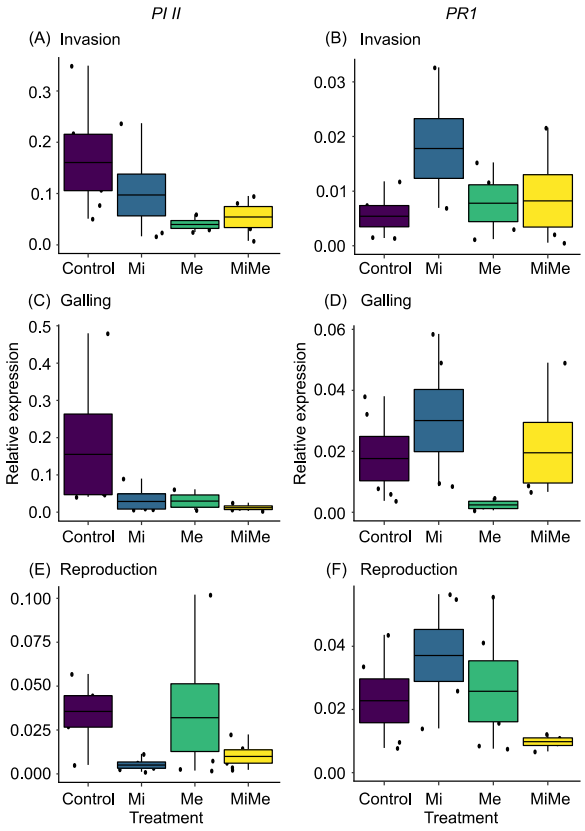


**Figure 2 Expression of the jasmonic acid (JA) marker gene *Proteinase inhibitor II* (*PI II*) and the salicylic acid (SA) marker gene *Pathogenesis-related protein 1* (PR1) in tomato leaves upon aboveground and belowground herbivory**. Relative expression of *PI II* and *PR1* genes were analyzed in leaves of tomato plants infected belowground with *Meloidogyne incognita* (Mi), or infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Expression values are normalized over the expression of the housekeeping gene *SIEF X14449* encoding for tomato *elongation factor 1α*. Boxplots indicate mean (±SEM, *n*=5) expression values of *PI II* (A,C,E) and *PR1* (B,D,F), measured at the nematodes' invasion (A,B), galling (C,D) or reproduction (E,F) stages. Different letters above the boxplots indicate significant differences ($P \leq 0.05$) in mean expression among treatments, determined by Tukeys HSD test after ANOVA.

**Effect of *Meloidogyne incognita* root infection on the aboveground accumulation of steroidal glycoalkaloids induced by *Macrosiphum euphorbiae***

Steroidal glycoalkaloids are important antiherbivore defense compounds in *Solanaceae* plants (Chowański et al., 2016). We first assessed the local effect of the aphid *Ma. euphorbiae* on the leaf concentrations of the steroidal glycoalkaloids *α*-dehydrotomatine and *α*-tomatine, and the expression of the steroidal glycoalkaloid-related genes *JRE4* (master transcriptional regulator in defense-related steroidal glycoalkaloids) and *GAME1* (encoding a UDP-Gal:tomatidine galactosyltransferase) when feeding on plants of different ages (Figure 3). Leaf feeding by the aphid *Ma. euphorbiae* led to a decrease in the concentrations of *α*-dehydrotomatine and *α*-tomatine, in young plants compared to controls (Figures 3A,B, green vs. purple boxplots, Table S6). In agreement, *Ma. euphorbiae* feeding on young plants significantly downregulated the expression of *JRE4* (Figure 3C, green vs. purple boxplot, Table S6). By contrast, *Ma. euphorbiae* feeding did not affect *GAME1* expression in young plants (Figure 3D, green vs. purple boxplot, Table S6). In the medium and old plants, infestation by *Ma. euphorbiae* did not alter the concentrations of *α*-dehydrotomatine and *α*-tomatine nor the expression of *JRE4* and *GAME1* compared to controls (Figure 3E-L, green vs. purple boxplots, Table S6). These findings show that the aphid *Ma. euphorbiae* represses the accumulation of *α*-dehydrotomatine and *α*-tomatine specifically when feeding on young plants.

We next studied the systemic impact of *M. incognita* root infection throughout the nematodes' infection cycle, on the leaf concentration of *α*-dehydrotomatine and *α*-tomatine, as well as on the expression levels of *JRE4* and *GAME1* genes. At the invasion stage, the leaf concentration of *α*-dehydrotomatine and *α*-tomatine decreased in *M. incognita* infected plants compared to control plants (Figure 3A,B, blue vs. purple boxplots, Table S6). Although not statistically significant ($p$=0.0892), we found a slight downregulation of *JRE4* in leaves of plants that were infected with *M. incognita* at the invasion stage (Figure 3C, blue vs. purple boxplot, Table S6). *M. incognita* at the invasion stage did not significantly affect *GAME 1* expression in leaves (Figure 3D, blue vs. purple boxplot, Table S6). At the *M. incognita* galling and reproduction stages, the leaf levels of *α*-dehydrotomatine and *α*-tomatine, and the expression of *JRE4* and *GAME 1* were similar in *M. incognita* infected plants and control plants (Figure 3G,H,K,L, blue vs. purple boxplots, Table S6). These results indicate that *M. incognita* triggers early and transient repression in the accumulation of *α*-dehydrotomatine and *α*-tomatine, specifically at the nematodes' invasion stage.

To check whether *M. incognita* root infection alters the repression of steroidal glycoalkaloid levels by the aphid *Ma. euphorbiae*, we compared plants challenged with *Ma. euphorbiae* alone to those co-infected with both *M. incognita* and *Ma. euphorbiae* at each of the nematodes' infection stages (Figure 3, yellow vs. green boxplots, Table S6). At the invasion stage, double-infected plants overall had higher *α*-dehydrotomatine and *α*-tomatine levels than plants infested by *Ma. euphorbiae* alone (Table S6, Mi*Me effect; Figure 3A,B, yellow vs. green boxplots). The expression of *JRE4* and *GAME1* in double infected

plants at the nematodes' invasion stage remained repressed and similar to that in plants challenged with *Ma. euphorbiae* alone (Figure 3C,D, yellow vs. green boxplots, Table S6). At the galling and reproduction stages of *M. incognita*, the concentrations of *α*-dehydrotomatine and *α*-tomatine, as well as the expression of *JRE4* and *GAME1* in double infected plants were similar to those plants challenged with *Ma. euphorbiae* alone (Figures 3E-L, yellow vs. green boxplots, Table S6). Our results indicate a moderate effect of *M. incognita* root infection on shoot steroidal glycoalkaloids patterns associated with the aphid *Ma. euphorbiae* feeding.

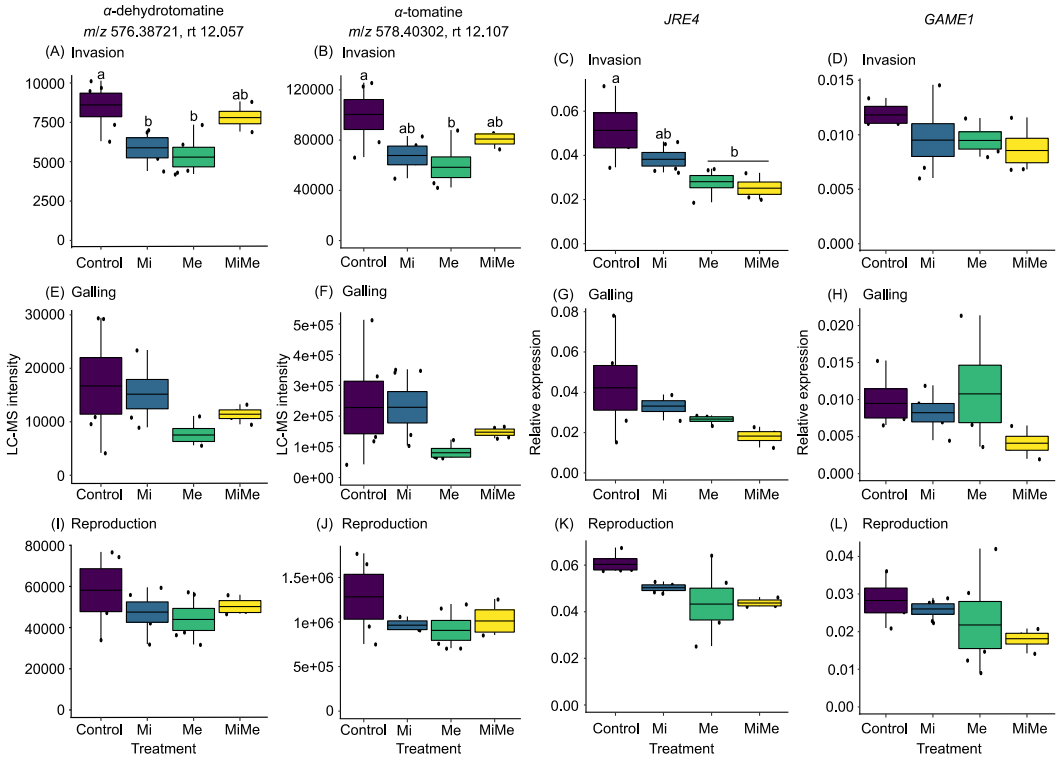


**Figure 3 Relative intensities of the *m*/*z* signals of the steroidal glycoalkaloids *α*-dehydrotomatine and *α*-tomatine and relative expression of glycoalkaloid-related metabolism genes *jasmonate-responsive ETHYLENE RESPONSE FACTOR 4* (*JRE4*) and *glycoalkaloid metabolism 1* (*GAME1)* in tomato leaves upon aboveground and belowground herbivory.** Mean intensities of *α*-dehydrotomatine (*m*/*z* 576.38721; rt 12.057 min) and *α*-tomatine (*m*/*z* 578.40302; rt 12.107 min) and expression of *JRE4* and *GAME1* in leaves tomato plants infected belowground with *Meloidogyne incognita* (Mi), or infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Boxplots are the mean (±SEM, *n*=5) of *α*-dehydrotomatine (A,E,I), *α*-tomatine (B,F,J), *JRE4* (C,G,K) and *GAME1* (D,H,L) measured at the nematodes' invasion (A,B,C,D), galling (E,F,G,H) and reproduction (I,J,K,L) stages. Different letters above the boxplots indicate significant ($P \leq 0.05$) differences in mean values between treatments, determined by Tukeys HSD test after ANOVA.

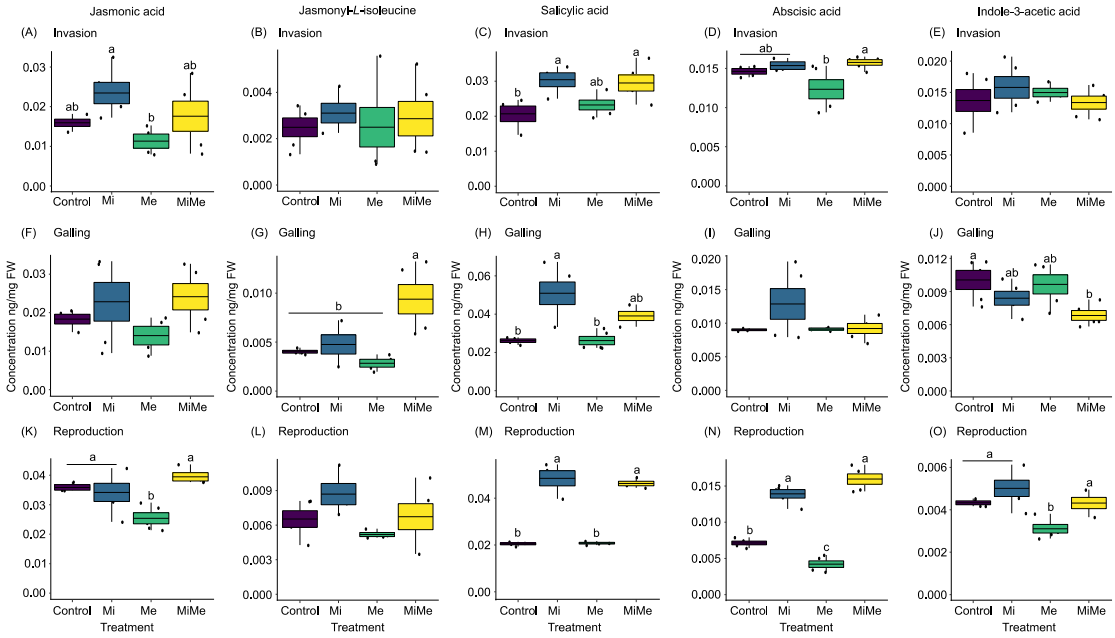


**Figure 4 Phytohormones concentrations in tomato roots upon aboveground and belowground herbivory.** Mean concentrations (ng/mg fresh weight) of phytohormones in roots of tomato infected belowground with *Meloidogyne incognita* (Mi), or infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Boxplots indicate the mean (±SEM, *n*=5) of jasmonic acid (A,F,K); jasmonyl-*L*-isoleucine (B,G,L); Indole-3- acetic acid (C,H,M); abscisic acid (D,I,N); salicylic acid (E,J,O) concentrations measured at the nematodes' invasion (A,B,C,D,E), galling (F,G,H,I,J) or reproduction (K,L,M,N,O) stages. Different lower-case letters above the boxplots indicate significant differences ($P \leq 0.05$) in mean values between treatments, determined by Tukeys HSD test after ANOVA.

### Impact of *Macrosiphum euphorbiae* leaf herbivory on root hormonal related responses triggered by *Meloidogyne incognita* infection

We started by analyzing the local impact of *M. incognita* on root phytohormonal-related responses throughout its infection cycle. The RKN *M. incognita* root infection neither affected the level of JA, JA-Ile, nor IAA in tomato roots, regardless of the infection cycle stage (Figure 4A,B,E,F,G,J,K,L,O, blue vs purple boxplots, Table S7). Remarkably, a main effect of *M. incognita* root infection was found for JA and JA-Ile levels (Table S7, Mi effect). However, *M. incognita* did not affect the expression of *PI II* compared to controls regardless of the infection stage (Figure 5A,C,E, blue vs. purple boxplots, Table S8). *M. incognita* root infection triggered the root accumulation of SA at all infection stages compared to controls (Figure 4C,H,M, blue vs. purple boxplots, Table S7). However, *M. incognita* root infection did not alter the expression of *PR1* gene compared to controls (Figure 5B,D,F, blue vs. purple boxplots, Table S8). We found similar ABA levels in control roots and *M. incognita* infected roots at the nematodes' invasion and

galling stages (Table S7, Mi effect) (Figure 4D, blue vs. purple boxplot). At the reproduction stage, *M. incognita* significantly increased the ABA concentrations compared to controls (Figure 4N, blue vs. purple boxplot, Table S7).

Leaf herbivory by the aphid *Ma. euphorbiae* did not systemically affect the root levels of JA, JA-Ile, ABA, or IAA when feeding on young or medium-age plants (Figure 4A,B,D,E,F,G,I,J green vs. purple boxplots, Table S7). When feeding on old plants, *Ma. euphorbiae* infestation led to a significant decrease in the root levels of JA, ABA, and IAA (Figure 4K,N,O, green vs. purple boxplots, Table S7). *Ma. euphorbiae* feeding, in general, did not affect the expression level of *PI II* and *PR1*, regardless of plant age. Only in medium-age plants, the expression levels of *PR1* decreased in the roots of plants challenged with *Ma. euphorbiae* (Figure 5D, green vs. purple boxplot, Table S8).

We finally assessed whether aphid feeding affected the phytohormonal root responses associated with *M. incognita* root infection (Figures 4,5, yellow vs. blue boxplots, Tables S7,S8). Roots of *M. incognita* had similar levels of JA, SA, ABA, and IAA as roots of plants challenged with *M. incognita* and *Ma. euphorbiae* (Figure 4A,C-F,H-K,M-N, yellow vs. blue boxplots). Similar to the SA levels, the expression of *PR1* in *M. incognita* infected roots did not differ from that in roots of double infected plants (Figure 5D, yellow vs. blue boxplots, Table S8). The levels of JA-Ile in the roots of double infected plants were higher compared to those found in roots of *M. incognita* infected roots, specifically at the nematode's galling stage (Figure 4G, yellow vs. blue boxplots, Table S7). By contrast, a higher expression level of *PI II* was found in roots of *M. incognita* infected plants compared to roots of plants infected with both herbivores at the nematodes' galling stage. At the invasion and reproduction stages, *PI II* expression was similar in *M. incognita* and double infected plants (Figure 5A,E, yellow vs. blue boxplots, Table S8).

**Effect of *Macrosiphum euphorbiae* feeding on root steroidal glycoalkaloids in plants with *Meloidogyne incognita***

We first analyzed the local impact of *M. incognita* on the root concentration of *α*-dehydrotomatine and *α*-tomatine, and the expression of *JRE4* and *GAME1* genes, throughout the nematode infection cycle. *M. incognita* root infection at the invasion and reproduction stages did not significantly affect the root level of *α*-dehydrotomatine and *α*-tomatine or the expression of *JRE4* and *GAME1* (Figure 6A-D,I-L, blue vs. purple boxplot, Table S9). Remarkably, at the nematodes' galling stage, *M. incognita* increased the level of *α*-dehydrotomatine and *α*-tomatine and the expression of *JRE4* and *GAME1* (Figure 6E-H, blue vs. purple boxplot, Table S9).

We then assessed the systemic impact of *Ma. euphorbiae* leaf herbivory on the root level of the steroidal glycoalkaloids. Leaf herbivory by *Ma. euphorbiae* did not affect the level of *α*-dehydrotomatine

and $\alpha$-tomatine, nor the expression of *JRE4* and *GAME1*, regardless of the plant age (Figure 6, green vs. purple boxplots, Table S9).

Finally, we analyzed whether *Ma. euphorbiae* feeding systemically affects the root levels of steroidal glycoalkaloids patterns associated with *M. incognita* root infection. In general, a similar level of $\alpha$-dehydrotomatine and $\alpha$-tomatine, and similar expression of *JRE4* and *GAME1* was found in roots of *M. incognita* infected plants and in the roots of double infected plants, regardless of the nematode infection stage (Figure 6, yellow vs. blue boxplots, Table S9). Only in the case of *JRE4* expression, a higher expression level was found in the roots of double infected plants compared to the roots of plants infected with *M. incognita* at the nematodes' reproduction stage (Figure 6K, yellow vs. blue boxplots, Table S9). Overall, these results reveal a minor effect of *Ma. euphorbiae* leaf herbivory on root steroidal glycoalkaloid patterns associated with the RKN *M. incognita* root infection.

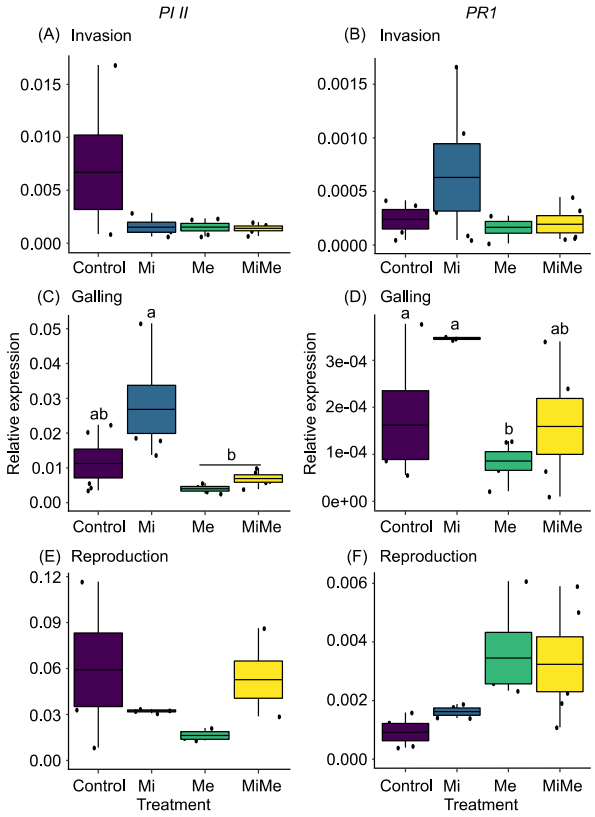


**Figure 5 Expression of the jasmonic acid (JA) marker gene *Proteinase inhibitor II* (*PI II*) and the salicylic acid (SA) marker gene *Pathogenesis-related protein 1* (PR1) in tomato roots upon aboveground and belowground herbivory**. Relative expression of *PI II* and *PR1* genes were analyzed in roots of tomato plants infected belowground with *Meloidogyne incognita* (Mi), infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Expression values are normalized over the expression of the housekeeping gene *SIEF X14449* encoding for tomato *elongation factor 1α*. Boxplots indicate mean (±SEM, *n*=5) expression values of *PI II* (A,C,E) and *PR1* (B,D,F) measured at the nematodes' invasion (A,B), galling (C,D) or reproduction (E,F) stages. Different lower-case letters above the boxplots indicate significant differences ($P \leq 0.05$) in mean expression among treatments, determined by Tukeys HSD test after ANOVA.

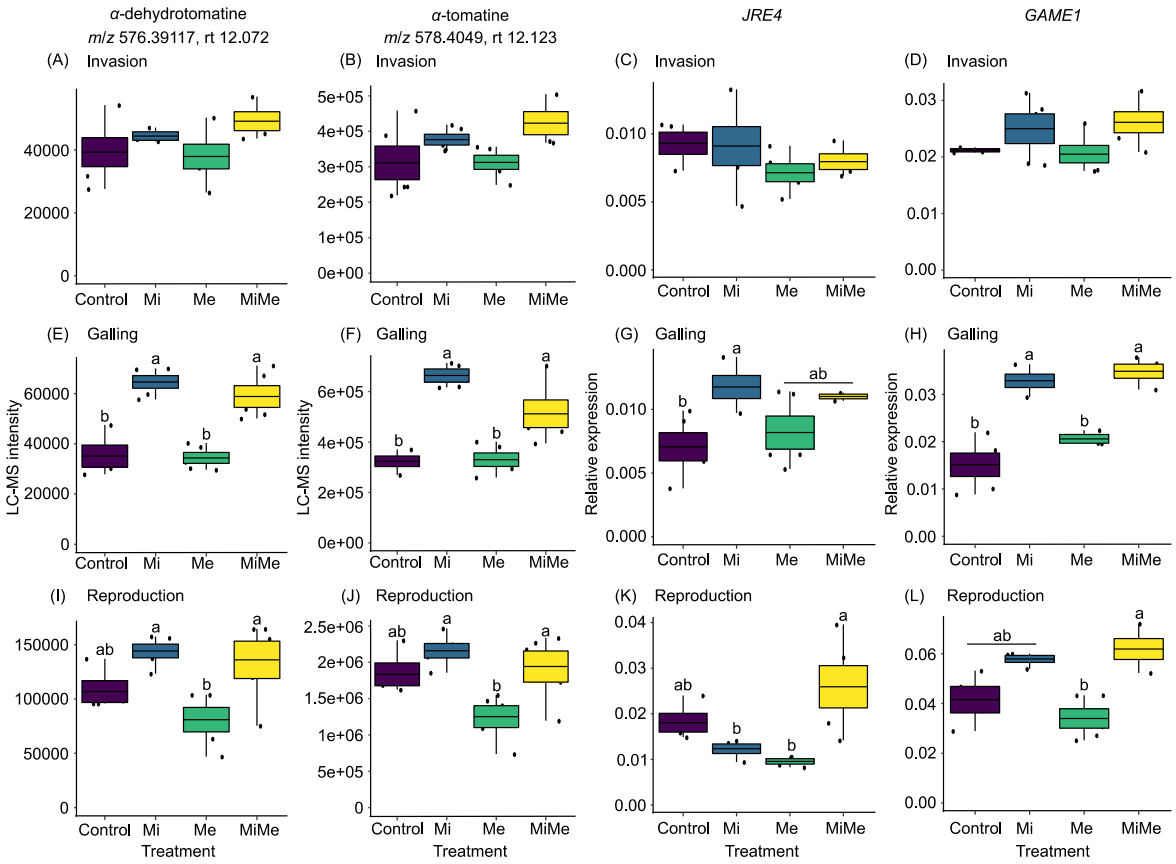


**Figure 6 Relative intensities of the *m/z* signals of the steroidal glycoalkaloids *α*-dehydrotomatine and *α*-tomatine and relative expression of glycoalkaloid-related metabolism genes *jasmonate-responsive ETHYLENE RESPONSE FACTOR 4* (*JRE4*) and *glycoalkaloid metabolism 1* (*GAME1)* in tomato roots upon aboveground and belowground herbivory.** Mean intensities of *α*-dehydrotomatine (*m/z* 576.38721; rt 12.057 min) and *α*-tomatine (*m/z* 578.40302; rt 12.107 min) and expression of *JRE4* and *GAME1* in roots of tomato plants infected belowground with *Meloidogyne incognita* (Mi), infested aboveground with *Macrosiphum euphorbiae* (Me) or with both herbivores (MiMe). Control = plants without herbivores. Boxplots indicate the mean (±SEM, *n*=5) of *α*-dehydrotomatine (A,E,I), *α*-tomatine (B,F,J) m/z intensities, or *JRE4* (C,G,K) and *GAME1* (D,H,L) relative expression measured at the nematodes' invasion (A,B,C,D), galling (E,F,G,H) or reproduction (I,J,K,L) stages. Different lower-case letters above the boxplots indicate significant differences ($P \leq 0.05$) in mean values between treatments, determined by Tukeys HSD test after ANOVA.

### The impact of *Meloidogyne incognita* root infection on *Macrosiphum euphorbiae* and the reverse effect of the aphid on the RKN

The reproduction of the aphid *Ma. euphorbiae* on *M. incognita* root infected plants did not differ compared to controls, regardless of the nematode infection cycle stage (Table S2). One the hand, we counted high number of root galls suggesting that the RKNs' development was not negatively affected (Table S1).

## DISCUSSION

Most of the studies on plant-mediated interactions between AG and BG herbivores via inducible responses focus on the impact of herbivory in one compartment on the induced responses in the other or opposite compartment (Erb et al., 2009; Kumar et al., 2016; Hoysted et al., 2017, 2018; Wang et al., 2017; Machado et al., 2018; van Dam et al., 2018). However, much less is known about how plants integrate sequential BG and AG attack, and the resulting concomitant induced responses in AG and BG organs of the same plant (Kutyniok and Muller, 2012; McCarville et al., 2012; Kammerhofer et al., 2015a). Here, by using tomato as a model plant, we studied how root infection by the RKN *M. incognita* affects the leaf responses triggered by the aphid *Ma. euphorbiae*, and the reciprocal impact of leaf herbivory by *Ma. euphorbiae* on root responses triggered by *M. incognita* infection. Moreover, because plant responses to *M. incognita* infection are tightly modulated during the nematodes' infection cycle (Kammerhofer et al., 2015b; Chapter 3 of this thesis), we studied the dynamics of the plant responses to the interaction between these two herbivores during the entire nematodes' infection cycle.

We found that the aphid feeding did not significantly affect phytohormonal signaling in leaves. In contrast to our results, several studies revealed that plants can activate the SA pathway upon attack by aphids, including *Ma. euphorbiae* (Mohase and van der Westhuizen, 2002; Chaman et al., 2003; Kuśnierczyk et al., 2008; Coppola et al., 2013). For instance, an increase in the expression of SA-responsive genes has been reported in *A. thaliana* upon the attack by *M. persicae* (Moran and Thompson, 2001) and of *Schizaphis graminum* on aphid-susceptible barley (Chaman et al., 2003). Moreover, the attack by *Ma. euphobiae*, *B. brassicae,* or *M. persicae* triggered the expression of both SA- and JA-responsive genes in Arabidopsis and tomato plants (de Ilarduya et al., 2003; Kuśnierczyk et al., 2008; Coppola et al., 2013). Such apparent discrepancies with our results may be partly explained by considering that, in a compatible interaction, and depending on the system under investigation, phloem feeders may antagonize the innate wound responses to make the plant a more suitable host (Walling, 2008). Indeed aphids are regarded as 'stealthy' feeders and can invoke minimal damage on the leaf tissues and thus no dramatic activation of the hormonal signaling pathways (Voelckel et al., 2004). In line with this, *Ma. euphorbiae* infestation reduced the leaf levels of the steroidal glycoalkaloids $\alpha$-dehydrotomatine and $\alpha$-tomatine and the expression of steroidal glycoalkaloid-related gene *JRE4*. *Solanum* alkaloids have a broad range of biological activity against insect herbivores, including aphids (Chowański et al., 2016). Thus, our results indicate the ability of *Ma. euphorbiae* to manipulate the secondary chemistry of their host plant. Along the same lines, previous studies showed that aphids, including *Ma. euphorbiae* and *M. perciase*, can decrease secondary metabolites as well as trigger the downregulation of a set of alkaloid biosynthesis genes in tomato and *A. thaliana* (Mewis et al., 2012; Coppola et al., 2013). It is well known that aphids contain effector proteins in their salivary secretions, which help to reduce the harmful effects of defenses induced against them (Hogenhout

and Bos, 2011; Kettles and Kaloshian, 2016). Interestingly, the aphid-triggered decrease in steroidal glycoalkaloids was specifically observed when the aphid fed on plants at the vegetative stage (young and medium age plants). By contrast, *Ma. euphorbiae* failed to counteract the steroidal glycoalkaloids- related responses in plants at the flowering stage (old plants), indicating that plant age and ontogeny are important factors determining the ability of *Ma. euphorbiae* to modulate plant defense responses. Following the failure of *Ma. euphorbiae* to counteract the steroidal glycoalkaloids-related responses in flowering plants, we found that the aphid performed poorly when feeding on those plants, compared to plants in the vegetative stage (Table S2). This suggests that the suppression of steroidal glycoalkaloids-related responses in local tissues can be important for the aphid's performance.

Whereas *Ma. euphorbiae* feeding did not induce phytohormonal responses locally in leaves, it systemically decreased the levels of JA, ABA, and IAA in roots, suggesting that this aphid might systemically alter the phytohormonal balance as well as the allocation of defenses between roots and shoots. It has been previously demonstrated that aphids can reduce aliphatic GS in the roots. This led to a shift in the aliphatic/indole GS ratio in systemic tissues, indicating that upon aphids attack the plants alter the allocation of defense compounds in a highly fine-tuned way (Kutyniok and Muller, 2012). Remarkably, the systemic impact of *Ma. euphorbiae* on root phytohormonal responses was observed when the aphid fed on flowering plants. Moreover, we also found a trend for a reduction of steroidal glycoalkaloids levels in roots when *Ma. euphorbiae* fed on flowering plants. This suggests that plant age and ontogeny are also important factors influencing the systemic effect of aphid leaf herbivory on root responses. It has been shown that after herbivory, plants prioritize the allocation of defenses to reproductive tissues over vegetative tissues (Chrétien et al., 2018). However, the ecological consequences of the decrease in the levels of phytohormones and glycoalkaloids triggered systemically by the aphid in the roots of the flowering plants remain unclear.

Root infection by the RKN *M. incognita* triggered an increase of SA levels locally in roots throughout the entire infection cycle. Similar to our results, the local accumulation of SA in roots upon the RKN infection has been demonstrated in several plant systems, including *A. thaliana*, rice, and tomato (Branch et al., 2004; Hamamouch et al., 2011; Kumari et al., 2016; Guo and Ge, 2017). The increased SA levels have been associated with elevated plant resistance against the nematodes (Molinari and Baser, 2010; Molinari et al., 2014). However, despite the increased SA levels, *M. incognita* performed well as we counted a high number of root-knots/galls (Table S1). The most parsimonious explanation is that RKNs can suppress or neutralize the responses via the utilization of effector proteins (Haegeman et al., 2012). Moreover, it has been shown that the effects of induced defense responses can be dose-dependent. Indeed, transgenic and mutant plants with reduced levels of SA were found more susceptible to nematode infection (Wubben et al., 2008; Nahar et al., 2011). Besides the increase in SA levels, we also found that the levels of ABA

increased specifically at the reproduction stage of the RKN *M. incognita*. It has been demonstrated that during RKN-plant interactions, ABA is associated with increasing the plant's susceptibility to RKN infection (Kyndt et al., 2017). Because the increase in ABA in this experiment occurred at the reproduction stage of the RKN *M. incognita*, we hypothesize that it enhances the susceptibility of the plants to newly hatching infective juveniles.

Besides the changes in phytohormone levels locally in roots, the RKN *M. incognita* also altered the steroidal glycoalkaloids-related response. In this case, specifically at the galling stage, the concentrations of the steroidal glycoalkaloid *α*-dehydrotomatine and *α*-tomatine and the expression of the steroidal glycoalkaloid related genes increased. In agreement with our results, an increase in α-tomatine levels has been reported in tomato plants infected by the RKN *M. incognita* and at the galling stage (Elliger et al., 1988). Although the induction of steroidal glycoalkaloids is associated with enhanced resistance to nematodes and other herbivores (Wang et al., 2012; Jang et al., 2015; Chowański et al., 2016; Garcia et al., 2018), their effects in the present study remain unclear.

By contrast to the strong and diverse local effects on root signaling, the RKN *M. incognita*, only affected SA signaling in leaves. Such a mild effect of root parasitic nematodes on systemic shoot tissues has been previously observed (Kutyniok and Muller, 2012). Indeed, we recently found that *M. incognita* infection did not directly affect JAs-related responses in tomato leaves, regardless of the nematodes' infection cycle stages (Chapter 2 of this thesis). Moreover, we found increased SA levels in leaves of *M. incognita*-plants only when the nematode was at the reproduction stage. This underscores the importance of the nematodes' life cycle stage for induction of defense in AG organs. *M. incognita* root infection reduced the steroidal glycoalkaloids-related responses in leaves systemically, although this effect was significant just for *α*-dehydrotomatine. Remarkably, the *M. incognita*-triggered repression of glycoalkaloids was specifically associated with the nematode's invasion stage. Plant-parasitic nematodes use an array of effectors that are essential for parasitizing their host (Haegeman et al., 2012). Although it is unclear how systemic repression of host plant defenses can benefit RKN parasitism, some of the nematode effectors are shown to suppress systemic signaling of defense responses in shoots (Kyndt et al., 2014). These results indicate that the RKNs can systemically cause at least subtle changes in major defense compounds of tomato.

Infection with the RKN *M. incognita* and the aphid *Ma. euphorbiae*, in general, did not affect the leaf phytohormonal profile associated with *Ma. euphorbiae* leaf feeding. However, RKNs at the reproduction stage also induced an increase in SA levels in plants that were also infested with the aphid *Ma. euphorbiae.* This indicates that the aphid was not able to counteract the burst of SA signaling triggered systemically by *M. incognita* root infection. Several studies demonstrated the master role of the SA pathway in plant defense against aphids (Mohase and van der Westhuizen, 2002; Morkunas et al., 2011). However,

the increased SA levels triggered the RKN did not affect the numbers of *Ma. euphorbiae* nymphs (Table S2). Several studies show that SA levels increase in shoots after nematode infection, but these changes differentially affect AG piercing-sucking insect herbivores. For example, an increase in SA concentration reduces the population abundance of *Bemisia tabaci* in tomato (Guo and Ge, 2017), whereas it does not affect the reproductive success of the green peach aphid (*M. perciae*) in potato (Hoysted et al., 2017). In another study, the delivery of pea aphid effector protein (Armet) activates the SA pathway in *N. benthamiana* and *Medicago truncatula* without adverse effects on the aphid's survival and reproduction (Cui et al., 2019).

*M. incognita* infection affected only slightly affected the steroidal glycoalkaloid-related responses triggered by the aphid *Ma. euphorbiae* feeding on plants at the vegetative stage. Indeed, the levels of *α*-dehydrotomatine and *α*-tomatine in leaves of double infected plants were in between the levels found in control and *Ma. euphorbiae* plants. This indicates a slight counteraction of the aphid's effects on steroidal glycoalkaloids when they co-occurred with the RKN *M. incognita*. Despite these mild effects (see Table S6), *M. incognita* root infection did not affect the reproduction of the aphid (Table S2). These results indicate that the counteractive effect on the steroidal glycoalkaloids was not enough to influence the aphid's performance.

In roots, the aphid *Ma. euphorbiae* did, in general, not affect the phytohormonal profile associated with *M. incognita* infection. Indeed, the increased SA levels triggered by *M. incognita* throughout its infection cycle were still evident when both the RKN *M. incognita* and the aphid *Ma. euphorbiae* co-occurred on the tomato plants. In analogy, *Ma. euphorbiae* did not interfere with the increased ABA levels triggered by *M. incognita* at the reproduction stage, even when *Ma. euphorbiae* infestation alone decreased ABA levels systemically in roots. These results indicate that the local effect of the RKN *M. incognita* on root phytohormonal-related responses cannot be overruled by the systemic effects of the aphid *Ma. euphorbiae* leaf herbivory. In accordance, in co-infected plants, *Ma. euphorbiae* was unable to systemically decrease the levels of JA, ABA, and IAA, as observed in plants that were infested with *Ma. euphorbiae* alone. In analogy, aphid feeding did not interfere with the increased steroidal glycoalkaloid levels triggered by *M. incognita* at the galling stage. This further corroborates that the local effect of *M. incognita* determined the plant responses regardless of the later arriving aphid *Ma. euphorbiae.* This phenomenon, where the first herbivore makes the host plant less reactive to the subsequent herbivore is called canalization. Canalization of plant defense responses by herbivores has been demonstrated in several systems, however, mostly in AG organs. For instance, in *S. dulcamara*, herbivory by the tortoise beetle (*Plagiometriona clavata*) did not alter the induced resistance elicited by the flea beetle (*Psylliodes affinis*) (Thaler et al., 2002; Viswanathan et al., 2007). Canalization of defense responses might occur via negative cross-talk between signaling pathways. This means that the first signaling pathway to be induced represses

the induction of the other pathways (Erb et al., 2012; Thaler et al., 2012). Based on our results, we could not tell whether the canalization effects on SA signaling and steroidal glycoalkaloid levels caused by the RKN *M. incognita* root infection was due to cross-talk or not. More studies are needed to provide more details.

In conclusion, we found that both the RKN *M. incognita* and the aphid *Ma. euphorbiae* triggered different local and systemic defense responses in tomato plants. When both herbivores co-occurred, the RKN *M. incognita* caused just mild systemic effects on the induced plant responses to the aphid herbivory in leaves. On the other hand, *M. incognita*-induced local root responses were not overruled by the systemic effect caused by *Ma. euphorbiae* leaf herbivory.

## DATA STATEMENT

The data underlying this study will be open access. We have deposited the data in iDiv data repository under the title, "Mbaluto C., Martinez-Medina A. and van Dam NM (2020) Induced defense response in tomato roots by root-knot nematode infestation at different life cycle stages and aboveground insect herbivory. iDiv Data Repository; http://idata.idiv.de/ddm/Data/ShowData/18xx". The DOI is currently under embargo until a decision on the manuscript is made.

## ACKNOWLEDGMENTS

All authors gratefully acknowledge the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118). We thank Dr. Zeger van Herwijnen of Rijk-Zwaan Breeding B.V (De Lier, The Netherlands), for providing us with the potato aphids. We also thank Dr. Fredd Vergara and Dr. Katharina Grosser for their technical support in molecular and chemical analysis. Crispus Mbaluto thanks the Deutscher Akademischer Austauschdienst (DAAD) for a doctoral research grant (research grant No: 91607343). Dr. Ainhoa Martínez-Medina further acknowledges funding from the program for attracting talent to Salamanca from the Fundación Salamanca Ciudad de Cultura y Saberes and Ayuntamiento de Salamanca.

## AUTHOR CONTRIBUTIONS

**CMM;** contributed to the conception of the idea, experimental design and conducting the experiments, processing of samples, data analysis, literature search and drafting the initial manuscript; **NMvD** and **AMM**; contributed to the conception of the idea, experimental designing, critical revisions of the draft manuscript and approval of the final manuscript for submission.

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## SUPPORTING INFORMATION

**Tables S1 Number of root galls induced by *Meloidogyne incognita* in tomato roots at different infection stages.** The number of induced root galls were counted in roots of tomato plants infected with *Meloidogyne incognita* (Mi) or co-infected with Mi and *Macrosiphum euphorbiae* (MiMe). Root galls were checked and counted at the nematodes galling, or reproduction stages. Data are means ± Standard error of mean per treatment (*n*= 9-10).

| Nematode infection stage | Treatments | |
|---|---|---|
| | Mi | MiMe |
| Galling | 121.70±0.94 | 132.30±0.96 |
| Reproduction | 299.13±1.67 | 279.13±2.30 |

**Table S2 Number of nymphs produced by apterous *Macrosiphum euphorbiae* adults on tomato plants.** The number of *M. euphorbiae* nymphs produced were counted on tomato plants without root herbivores (Control) and on plants infected with *Meloidogyne incognita* (Mi). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. The counting was done after allowing the adult female aphids to infested the tomato plants for three days. Data are means±SEM. Statistical analysis were done with student *t-test* (*t-values*, df, and *p-values* are shown) per each nematode infection stage and per treatment (*n*=5). The data shown were obtained from two independent experiments.

| *M. incognita* infection stages | Treatment | | Student *t-test* results | | |
|---|---|---|---|---|---|
| | Control | Mi | T-value | Df | P-value |
| Invasion | 12.6±0.86 | 10.6±1.03 | 0.98533 | 8 | 0.3533 |
| Galling | 14.2±1.66 | 15.2±0.91 | -0.31039 | 8 | 0.7642 |
| Reproduction | 4.0±1.37 | 2.8±1.36 | 0.75295 | 8 | 0.4731 |

§; T: T- statistics, Df: degrees of freedom, P: probability value.

**Table S3 List of primers sequences used for qRT-PCR**

| Target gene | Hormone pathway | Primer sequences (5'-3') |
|---|---|---|
| *Proteinase inhibitor II (PI II)*[a] | Jasmonic acid (JA) | Fw: GAAAATCGTTAATTTATCCCAC<br>Rv: ACATACAAACTTTCCATCTTTA |
| *Pathogen-related Protein 1 (PR1)*[a] | Salicylic acid (SA) | Fw: GTGGGATCGGATTGATATCCT<br>Rv: CCTAAGCCACGATACCATGAA |
| *Jasmonate-responsive ETHYLENE RESPONSE FACTOR (ERF) transcription factor (JRE4)*[b] | coordinate transcription of metabolic genes in steroidal glycoalkaloids biosynthesis | Fw: TGTTTCCTCCGGTGTTACGG<br>Rv: CGATTTTTTCGAAACTCTTTCC |
| *GLYCOALKALOID METABOLISM 1 (GAME1)*[b] | convert acetyl-coenzyme A (*acetyl*-CoA) to steroidal glycoalkaloids | Fw: TTGCCGGATGTTCCATGATCG<br>Rv: CTAATGAAGAAACAGCGTCCTGG |
| *SIEF X14449* (Housekeeping gene)[a] | Elongation factor-1$\alpha$ | Fw: GATTGGTGGTATTGGAACTGTC<br>Rv: AGCTTCGTGGTGCATCTC |

[a]Martínez-Medina et al. (2013); [b]Abdelkareem et al. (2017); Fw: foward, Rv: reverse.

[b]Abdelkareem, A., Thagun, C., Nakayasu, M., Mizutani, M., Hashimoto, T., and Tsubasa Shoji (2017). Jasmonate-induced biosynthesis of steroidal glycoalkaloids depends on COI1 proteins in tomato. *Biochem. Biophys. Res. Commun.* 489, 206–210. doi:10.1016/j.bbrc.2017.05.132.

[a]Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M. J., Jung, S. C., Pascual, J. A., and Pozo, M. J. (2013). Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Front. Plant Sci.* 4, 1–12. doi:10.3389/fpls.2013.00206.

**Table S4 ANOVA table of phytohormone concentrations in tomato leaves upon aboveground and belowground herbivory.** Concentrations of jasmonyl-*L*-isoleucine (JA-Ile), salicylic acid (SA), abscisic acid (ABA), and indole-3-acetic acid (IAA) were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or infested with *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Leaf samples were taken 24 hours after infestation by *M. euphorbiae*. Data are the mean ± standard error ($n$=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Hormone measured | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| JA-Ile | Mi | 1,13 | 1.2325 | 0.2870 | 1,16 | 0.0871 | 0.7717 | 1,13 | 1.0657 | 0.3207 |
| | Me | 1,13 | 0.1341 | 0.7201 | 1,16 | 0.1242 | 0.7291 | 1,13 | 0.0248 | 0.8773 |
| | Mi*Me | 1,13 | 0.0949 | 0.7629 | 1,16 | 0.2838 | 0.6016 | 1,13 | 0.1412 | 0.7131 |
| SA | Mi | 1,11 | 1.9040 | 0.1950 | 1,16 | 3.8485 | 0.0674 | 1,16 | 88.8546 | **<0.000** |
| | Me | 1,11 | 0.3261 | 0.5795 | 1,16 | 0.7695 | 0.3934 | 1,16 | 0.0823 | 0.7779 |
| | Mi*Me | 1,11 | 1.6248 | 0.2287 | 1,16 | 0.0115 | 0.9160 | 1,16 | 0.4982 | 0.4904 |
| ABA | Mi | 1,16 | 0.0001 | 0.9906 | 1,14 | 0.3377 | 0.5704 | 1,15 | 1.0913 | 0.3127 |
| | Me | 1,16 | 0.1140 | 0.7400 | 1,14 | 1.2107 | 0.2897 | 1,15 | 2.8231 | 0.1136 |
| | Mi*Me | 1,16 | 0.2324 | 0.6363 | 1,14 | 0.0005 | 0.9817 | 1,15 | 4.1909 | 0.0586 |
| IAA | Mi | 1,16 | 0.5851 | 0.4555 | 1,15 | 0.6435 | 0.4350 | 1,15 | 5.8667 | **0.0286** |
| | Me | 1,16 | 0.0891 | 0.7692 | 1,15 | 2.4098 | 0.1414 | 1,15 | 6.2769 | **0.0243** |
| | Mi*Me | 1,16 | 0.0494 | 0.8270 | 1,15 | 0.2443 | 0.6283 | 1,15 | 0.0209 | 0.8869 |

§: Df(n,d): degrees of freedom (numerator, and denominator), F: F test value, P: probability value.

**Table S5 ANOVA table of expression of defense marker genes in tomato leaves upon aboveground and belowground herbivory.** The expression levels of *Proteinase inhibitors II (PI II)* and *Pathogen-related protein 1 (PR1)* were determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or infested with *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Leaf samples were taken 24 hours after infestation by *M. euphorbiae*. Data are the mean ± standard error ($n$=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold

| Marker gene | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df(n,d) | F | P | Df(n,d) | F | P | Df(n,d) | F | P |
| *PI II* | Mi | 1,14 | 0.5212 | 0.4822 | 1,11 | 1.6052 | 0.2313 | 1,16 | 5.8802 | **0.0275** |
| | Me | 1,14 | 4.1992 | 0.0597 | 1,11 | 1.2364 | 0.2899 | 1,16 | 0.0041 | 0.9497 |
| | Mi*Me | 1,14 | 0.9449 | 0.3475 | 1,11 | 0.8118 | 0.3869 | 1,16 | 0.1507 | 0.7030 |
| *PR1* | Mi | 1,13 | 2.9305 | 0.1107 | 1,13 | 2.6094 | 0.1302 | 1,15 | 0.0001 | 0.9910 |
| | Me | 1,13 | 0.7033 | 0.4168 | 1,13 | 1.9745 | 0.1834 | 1,15 | 2.1335 | 0.1647 |
| | Mi*Me | 1,13 | 2.3199 | 0.1517 | 1,13 | 0.0656 | 0.8019 | 1,15 | 3.8612 | 0.0682 |

§: Df(n,d): degrees of freedom (numerator, and denominator); F: F test value, P: probability value.

**Table S6 ANOVA table of the *m/z* intensities of steroidal glycoalkaloids and expression of glycoalkaloids metabolism genes in tomato leaves upon aboveground and belowground herbivory.** LC-MS intensities of the steroidal glycoalkaloids (*α*-dehydrotomatine and *α*-tomatine) and expression glycoalkaloids metabolism (GAME) genes (*jasmonate-responsive ETHYLENE RESPONSE FACTOR 4 (JRE4)* and *glycoalkaloid metabolism 1 (GAME1)*) were determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or infested with *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Leaf samples were taken 24 hours after infestation by *M. euphorbiae*. Data are the mean ± standard error (*n*=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Steroidal glycoalkaloid and GAME genes | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| *α*-dehydro tomatine | Mi | 1,14 | 0.0279 | 0.8698 | 1,14 | 0.0652 | 0.8021 | 1,13 | 0.1885 | 0.6713 |
| | Me | 1,14 | 2.3958 | 0.1440 | 1,14 | 3.5299 | 0.0813 | 1,13 | 0.9257 | 0.3535 |
| | Mi*Me | 1,14 | 16.5715 | **0.0011** | 1,14 | 0.6191 | 0.4445 | 1,13 | 1.5605 | 0.2336 |
| *α*-tomatine | Mi | 1,13 | 0.5865 | 0.4575 | 1,14 | 0.2816 | 0.6040 | 1,11 | 0.3329 | 0.5756 |
| | Me | 1,13 | 4.2296 | 0.0604 | 1,14 | 3.9982 | 0.0653 | 1,11 | 1.5404 | 0.2404 |
| | Mi*Me | 1,13 | 8.0747 | **0.0139** | 1,14 | 0.3371 | 0.5708 | 1,11 | 1.5824 | 0.2345 |
| *JRE4* | Mi | 1,14 | 3.3357 | 0.0892 | 1,13 | 1.6179 | 0.2257 | 1,12 | 1.1018 | 0.3146 |
| | Me | 1,14 | 17.1693 | **0.0010** | 1,13 | 4.8287 | **0.0467** | 1,12 | 7.1422 | **0.0203** |
| | Mi*Me | 1,14 | 1.3278 | 0.2685 | 1,13 | 0.0018 | 0.9671 | 1,12 | 1.2491 | 0.2856 |
| *GAME1* | Mi | 1,12 | 1.6585 | 0.2221 | 1,13 | 2.9759 | 0.1082 | 1,14 | 0.5531 | 0.4694 |
| | Me | 1,12 | 1.6088 | 0.2287 | 1,13 | 0.4974 | 0.4931 | 1,14 | 3.3116 | 0.0902 |
| | Mi*Me | 1,12 | 0.3116 | 0.5869 | 1,13 | 1.4866 | 0.2444 | 1,14 | 0.0297 | 0.8657 |

§: Df(n,d): degrees of freedom (numerator, and denominator); F: F test value, P: probability value.

**Table S7 ANOVA table of phytohormone concentrations in tomato roots upon aboveground and belowground herbivory.** Concentrations of jasmonic acid (JA), jasmonyl-*L*-isoleucine (JA-Ile), salicylic acid (SA), abscisic acid (ABA), and indole-3-acetic acid (IAA) were measured in roots of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Roots samples were taken 24 hours after leaf infestation by *M. euphorbiae*. Data are the mean ± standard error ($n$=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Hormone measured | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| JA | Mi | 1,14 | 6.1134 | **0.0269** | 1,14 | 3.9939 | 0.0655 | 1,13 | 8.6512 | **0.0115** |
| | Me | 1,14 | 3.6682 | 0.0761 | 1,14 | 0.1050 | 0.7507 | 1,13 | 0.6752 | 0.4261 |
| | Mi*Me | 1,14 | 0.0486 | 0.8287 | 1,14 | 0.5877 | 0.4560 | 1,13 | 11.3381 | **0.0051** |
| JA-Ile | Mi | 1,15 | 0.5300 | 0.4778 | 1,13 | 13.4672 | **0.0028** | 1,14 | 4.6329 | **0.0493** |
| | Me | 1,15 | 0.0281 | 0.8691 | 1,13 | 3.2875 | 0.0930 | 1,14 | 3.6727 | 0.0760 |
| | Mi*Me | 1,15 | 0.0333 | 0.8577 | 1,13 | 7.8327 | **0.0151** | 1,14 | 0.1269 | 0.7270 |
| SA | Mi | 1,14 | 15.2849 | **0.0016** | 1,14 | 25.3685 | **0.0002** | 1,12 | 245.7204 | **<0.000** |
| | Me | 1,14 | 0.1572 | 0.6977 | 1,14 | 2.4982 | 0.1363 | 1,12 | 0.3275 | 0.5777 |
| | Mi*Me | 1,14 | 0.7320 | 0.4066 | 1,14 | 2.5434 | 0.1331 | 1,12 | 0.5340 | 0.4790 |
| ABA | Mi | 1,13 | 8.4548 | **0.0122** | 1,12 | 1.4992 | 0.2443 | 1,16 | 303.8174 | **<0.000** |
| | Me | 1,13 | 1.6874 | 0.2165 | 1,12 | 2.0954 | 0.1734 | 1,16 | 0.7011 | 0.4147 |
| | Mi*Me | 1,13 | 2.6992 | 0.1244 | 1,12 | 1.3676 | 0.2649 | 1,16 | 21.817 | **0.0003** |
| IAA | Mi | 1,15 | 0.0592 | 0.8111 | 1,16 | 9.5523 | **0.0070** | 1,14 | 11.7829 | **0.0040** |
| | Me | 1,15 | 0.2280 | 0.6399 | 1,16 | 1.8358 | 0.1943 | 1,14 | 12.0715 | **0.0037** |
| | Mi*Me | 1,15 | 1.6313 | 0.2209 | 1,16 | 0.6431 | 0.4343 | 1,14 | 0.9331 | 0.3504 |

§: Df(n,d): degrees of freedom (numerator, and denominator); F: F test value, P: probability value.

**Table S8 ANOVA table of expression of defense marker genes in tomato roots upon aboveground and belowground herbivory.** The expression levels of *Proteinase inhibitors II (PI II)* and *Pathogen-related protein 1 (PR1)* were determined in roots of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or infested with *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Roots samples were taken 24 hours after leaf infestation by *M. euphorbiae*. Data are the mean ± standard error ($n$=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold

| Marker gene | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| *PI II* | Mi | 1,14 | 2.2809 | 0.1532 | 1,15 | 4.9210 | **0.0424** | 1,11 | 0.0262 | 0.8744 |
| | Me | 1,14 | 2.8110 | 0.1158 | 1,15 | 10.3194 | **0.0058** | 1,11 | 0.3618 | 0.5597 |
| | Mi*Me | 1,14 | 2.5846 | 0.1302 | 1,15 | 2.1073 | 0.1672 | 1,11 | 4.6506 | 0.0540 |
| *PR1* | Mi | 1,14 | 1.2226 | 0.2875 | 1,13 | 5.3753 | **0.0374** | 1,13 | 0.0955 | 0.7622 |
| | Me | 1,14 | 2.1424 | 0.1654 | 1,13 | 6.0000 | **0.0292** | 1,13 | 8.1719 | **0.0134** |
| | Mi*Me | 1,14 | 0.9070 | 0.3571 | 1,13 | 1.1360 | 0.3059 | 1,13 | 0.4030 | 0.5365 |

§: Df(n,d): degrees of freedom (numerator, and denominator); F: F test value, P: probability value

**Table S9 ANOVA table of the *m/z* intensities of steroidal glycoalkaloids and expression of glycoalkaloids metabolism genes in tomato roots upon aboveground and belowground herbivory**. LC-MS intensities of the steroidal glycoalkaloids (*α*-dehydrotomatine and *α*-tomatine) and expression glycoalkaloids metabolism (GAME) genes (*jasmonate-responsive ETHYLENE RESPONSE FACTOR 4 (JRE4)* and *glycoalkaloid metabolism 1 (GAME1)*) were determined in roots of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or infested with *Macrosiphum euphorbiae* (Me) alone, or co-infected with both herbivores (MiMe). In co-infected plants, infestation with *M. euphorbiae* was performed at the nematodes' invasion, galling, or reproduction stages. Root samples were taken 24 hours after leaf infestation by *M. euphorbiae*. Data are the mean ± standard error (*n*=5). Data were analyzed using Two-way ANOVA linear model consisting of *M. incognita* (Mi), *M. euphorbiae* (Me) and their interaction (Mi*Me) as model explanatory factors. Difference between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold

| Steroidal glycoalkaloid and GAME genes | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| *α*-dehydro tomatine | Mi | 1,15 | 0.6241 | 0.4418 | 1,13 | 8.4090 | **0.0124** | 1,14 | 42.0503 | **<0.000** |
| | Me | 1,15 | 0.6020 | 0.4499 | 1,13 | 0.1475 | 0.7071 | 1,14 | 8.4808 | **0.0114** |
| | Mi*Me | 1,15 | 2.0733 | 0.1704 | 1,13 | 6.2700 | **0.0264** | 1,14 | 1.9325 | 0.1862 |
| *α-t*omatine | Mi | 1,15 | 7.5346 | **0.0150** | 1,14 | 45.4045 | **<0.000** | 1,15 | 10.1773 | **0.0061** |
| | Me | 1,15 | 0.5137 | 0.4846 | 1,14 | 3.7155 | 0.0745 | 1,15 | 5.7338 | **0.0301** |
| | Mi*Me | 1,15 | 0.5076 | 0.4871 | 1,14 | 4.4095 | 0.0543 | 1,15 | 1.2597 | 0.2793 |
| *JRE4* | Mi | 1,14 | 0.0939 | 0.7637 | 1,13 | 11.5258 | **0.0048** | 1,13 | 3.8537 | 0.0714 |
| | Me | 1,14 | 2.7677 | 0.1184 | 1,13 | 0.1063 | 0.7496 | 1,13 | 1.0835 | 0.3169 |
| | Mi*Me | 1,14 | 0.2627 | 0.6163 | 1,13 | 0.6780 | 0.4251 | 1,13 | 13.4283 | **0.0029** |
| *GAME1* | Mi | 1,14 | 5.6853 | **0.0318** | 1,12 | 69.5573 | **<0.000** | 1,13 | 31.3507 | **<0.000** |
| | Me | 1,14 | 0.0310 | 0.8628 | 1,12 | 3.5265 | 0.0849 | 1,13 | 0.2647 | 0.6155 |
| | Mi*Me | 1,14 | 0.1896 | 0.6699 | 1,12 | 0.7703 | 0.3974 | 1,13 | 2.0717 | 0.1737 |

§: Df(n,d): degrees of freedom (numerator, and denominator); F: F test value, P: probability value.

# CHAPTER 5

# Discussion and conclusion

**Introduction**

Induced plant responses to AG and BG herbivores, can propagate to systemic tissues and underlie the AG-BG plant-mediated interactions (Erb et al., 2008; McCarville et al., 2012; Kutyniok and Müller, 2013; Kammerhofer et al., 2015; Biere and Goverse, 2016). The outcomes of these AG-BG plant-mediated interactions can be variable, and the factors and mechanisms underlying the variations remain unclear. The studies in this thesis focus on the role of phytohormonal signaling and expression of secondary metabolites in mediating the interaction between the BG feeding root-knot nematode (RKN: *Meloidogyne incognita*) on AG feeding insect herbivores (the caterpillar *Spodoptera exigua* and the aphid *Macrosiphum euphorbiae*) and the reciprocal effects of the AG insect herbivores on root responses triggered by the RKN throughout its infection cycle. In this chapter, I will elaborate on the findings in the context of; (1) interactions between AG and BG feeding herbivores, (2) mechanisms that shape AG-BG plant-mediated interactions, and (3) linkage of the induced systemic responses under sequential AG-BG attack to insect communities. Finally, I give conclusions and prospects for further research.

**Interaction between aboveground and belowground herbivores via induced systemic responses**

Over the last 3-4 decades, the scientific interest in plant-herbivore interactions shifted from AG and BG compartments in isolation to multitrophic scenarios crossing the AG-BG interface. Often plants are attacked by several species of herbivores that interact with each other at spatial and temporal scales (Erb et al., 2008; Wondafrash et al., 2013; Papadopoulou and van Dam, 2017; Heinen et al., 2018). Attack on one compartment can result in the induction of systemic responses in the other compartment, and this may influence the behavior and performance of subsequent herbivores. Following these lines, changes in primary and secondary metabolites are demonstrated to underlie these interactions (Gange and Brown, 1989; Wackers and Bezemer, 2003; Kaplan et al., 2008a, 2009; Erb et al., 2009; Kutyniok and Muller, 2012; Kutyniok and Müller, 2013; Kumar et al., 2016; van Dam et al., 2018; Karssemeijer et al., 2020). In these studies, both positive and negative effects on the performance of AG and BG herbivores have been demonstrated. As an example of negative effects, leaf chewing *S. exigua* and *S. frugiperda* gained less weight after feeding on potato plants suffering from root herbivory by the Guatemalan moth *(Tecia solanivora)* (Kumar et al., 2016). Shoot feeding aphids, including *Brevicoryne brassicae* and *Myzus perciae* were shown to prefer *Brassica nigra* and potato plants root infected by the parasitic nematodes *M. hapla* and *Globodera pallida* respectively (Hoysted et al., 2017; van Dam et al., 2018). On the other hand, AG herbivores can positively and negatively affect BG feeding herbivores. For example, clipping to mimic herbivory was found to increase the abundance of root-feeding nematodes in several grass species (Wang et al., 2017). Infestation by *M. persicae* AG diminishes the hatch potential of the cyst nematode *Globodera*

*pallida* cysts (Hoysted et al., 2018). Leaf feeding by diamondback moth (*Plutella xylostella*) strongly attenuates the performance of cabbage root fly larvae *Delia radicum* (Karssemeijer et al., 2020).

These studies suggest that the combination of different herbivory feeding styles (chewing vs. piercing-sucking), as well as the temporal variations (sequence of arrival), are some of the factors underlying the suitability of damaged plant to the subsequent herbivore (Erb et al., 2011; Wondafrash et al., 2013; Kafle et al., 2017). Keeping these factors in mind, I investigated whether the infection cycle stages of RKNs influences the plant's response to AG insect herbivores (chewing vs. piercing-sucking). Also, I studied the reciprocal effects of the AG insect herbivores on root-induced responses by the RKN. The results obtained here revealed that the impact of the RKN on AG insect herbivores varies depending on the RKN infection cycle, indicating that the RKN infection cycle is an important factor to consider when designing studies on AG-BG interactions involving them. I found that root infection by the RKN *M. incognita* enhanced the performance of leaf chewer *S. exigua* only during the nematodes' galling stage (Chapter 2), but had no effect at any life cycle stage on the performance of the phloem-feeder *Ma. euphorbiae* (Chapter 4). These results demonstrate that, in addition to the RKN infection cycle, the feeding mode of AG insect herbivore is also important for the interaction outcome. I further found that the AG insect herbivores differed in their systemic effect on RKN induced root responses. The caterpillar reduced the JA levels, while the aphid did not alter the induced root responses by the RKN (Chapters 3,4). Although I did not evaluate the effect of the caterpillar and aphid feeding on the performance of the RKN, other studies showed that AG insect herbivores including chewers, piercing-sucking insect herbivores, shoot elicitation with JA or MeJA or mechanical damage could positively or negatively affect the abundance and performance of parasitic root nematodes (Bhattarai et al., 2008; Vasyukova et al., 2009; Wang et al., 2017; Hoysted et al., 2018; Machado et al., 2018). Indeed, in Annex 1, I present a study in which I collaborated, showing the negative effect of AG herbivory by the caterpillar *Manduca sexta* on the performance of the RKN *M. incognita*. Overall, my studies demonstrate that for plant-mediated interactions between RKN and AG insect herbivores, the nematodes' infection cycle is an essential factor that influences the outcome of the interaction. In the next section, I discuss the changes in phytohormonal pathways and expression of secondary metabolites as the mechanisms shaping these AG-BG interactions.

**Mechanisms shaping plant-mediated aboveground and belowground interactions**

Among the mechanisms commonly invoked to explain the interaction between spatially or temporally separated organisms are the activation of defense-related pathways and the resultant expression of secondary metabolites (Wondafrash et al., 2013; Lazebnik et al., 2014; Chowański et al., 2016). Indeed, to cope with herbivory, plants evolved phytohormone-dependent defense signals and transcriptional responses with considerable specificity for the attacking herbivore (Walling, 2000; Diezel et al., 2009; Stam et al.,

2014). The JA and SA pathways play a central role in local and systemic induction of responses induced by chewers (e.g., caterpillars) and piercing-sucking herbivores (e.g., aphid, nematodes), respectively (Walling, 2000; Diezel et al., 2009; Guo and Ge, 2017; van Dam et al., 2018). Moreover, other hormones such as ABA, ethylene, and auxins play a crucial modulatory role in induced defenses against herbivores (Bari and Jones, 2009; Erb et al., 2012; Kammerhofer et al., 2015). Given that BG feeding nematodes and AG caterpillars and aphids can systemically influence each other, I investigated whether RKNs can affect the phytohormonal-related responses triggered by these AG insect herbivores, as well as the reciprocal effects of the AG insect herbivores on the RKN-induced root responses. As the plant responses to the RKN change in the course of root infection, I further studied the interaction between the RKN and AG insect herbivores at different stages of the nematodes' infection cycle, i.e., at the invasion, galling, and reproduction stages.

The results obtained showed that RKN infection increased root JA, SA, and ABA levels as well as shoot SA levels, but it did not affect the expression of the SA- nor JA-responsive marker genes. The root JA and ABA levels increased most significantly at the reproductions stage, while SA levels increase locally in roots throughout the infection cycle and systemically in leaves only at the reproduction stage (Chapters 3,4). These results correspond with other studies reporting the induction of JA, SA, and ABA in roots and shoots after root infection by RKNs (Fan et al., 2014; Guo and Ge, 2017; Kyndt et al., 2017; Seiml-Buchinger et al., 2019). The lack of effect on the expression of JA and SA responsive genes locally in roots and systemically in leaves contrasts with other studies (Fan et al., 2014; Molinari et al., 2014; Seiml-Buchinger et al., 2019). Although I do not have a specific explanation for such apparent discrepancies, they could be related to differences in the study systems. The antagonistic interaction between JA and SA is well recognized (Thaler et al., 2002; Pieterse et al., 2009). However, it is not surprising for RKN to induce both JA and SA in roots without evident antagonistic cross-talk between them. Indeed several studies demonstrate that induction of JA and SA coordinates resistance or susceptibility of the host plant to RKN (Bhattarai et al., 2008; Hamamouch et al., 2011; LiNing et al., 2011; Zinov'eva et al., 2013; Fan et al., 2014; Molinari et al., 2014). The dynamics of the JA and SA pathways in the RKN infection process can be modulated by the effector proteins secreted by the nematode (Haegeman et al., 2012). Besides the roles of JA and SA signaling, ABA signaling can increase the plant's susceptibility to RKN infection (Kyndt et al., 2017). Here, the increase in ABA levels occurred mostly at the reproduction stage of the RKN, I therefore hypothesized that, such increase enhances the susceptibility of the plants to newly hatching infective juveniles.

I further explored phytohormonal-related responses triggered locally and systemically by the AG insect herbivores (the caterpillar *S. exigua* and the aphid *Ma. euphorbiae*). Because in my experimental set-ups I used the RKN infection cycle stage to time the experiments, plants assigned for infestation with only

AG insect herbivores (and their respective control plants) had different ages that coincided with the RKN life cycle stages. These included: 4.8 weeks (young plants; =RKN invasion stage); ~6 weeks (medium-aged plants =RKN galling stage); and 8 weeks (old plants =RKN reproduction stage). The results obtained showed that caterpillar feeding triggered a general increase in JAs (OPDA, JA, and JA-Ile), SA and ABA levels, as well as the JAs-related marker genes locally in shoots, while systemically in roots, only the JAs marker genes were upregulated (Chapters 2-4, and Figure S1). Aphid feeding neither affected phytohormone levels nor their marker genes locally in shoots. In contrast, aphid feeding suppressed JA, ABA, and the auxin (Indole-3-acetic acid) levels and downregulated the expression of the SA marker gene *PR1* systemically in roots (Chapter 4). Remarkably, the effect of the AG insect herbivores on the phytohormones and the related marker genes in local and systemic tissues varied from young (vegetative stage) to old (flowering stage) plants. For example, caterpillar feeding increased the shoot level of JAs locally in leaves of plants at the vegetative stage, while it did not significantly affect JAs when feeding on plants at the flowering stage. By contrast, feeding by the caterpillar on flowering plants triggered the systemic root expression of the JA-marker gene *LapA* (*leucine aminopeptidase A*) (Chapter 3). On the other hand, feeding by aphid decreased the levels of JA, ABA, and IAA systemically in roots of the flowering plants, while it did not affect the phytohormonal-related responses in plants at the vegetative stage. These results indicate the influence of plant age and ontogeny in the local and systemic responses triggered by insect herbivores. Accordingly, previous studies have demonstrated the influence of the plant age and ontogeny in the induction of defense. For example, shoot herbivory on *Plantago lanceolata* L. increases the concentration of iridoid glycosides in roots, twice as high in mature plants compared to young plants (Quintero and Bowers, 2011, 2012).

Cross-talk between phytohormones can underlie the outcome of AG-BG interactions. For instance, root infection by the RKN *M. hapla* enhances the JA pathway and reduces the SA pathway, which increases the preference of the aphid *B. brassicae* to *M. hapla* infected *B. nigra* plants over controls (van Dam et al., 2018). It has been demonstrated that the outcome of cross-talk depends on the specific combination of herbivore species, their feeding guilds, the sequence of arrival, and the type of damage (van Dam et al., 2018; Davidson-Lowe et al., 2019). However, these factors stem from studies investigating interactions in AG and BG independently or one direction in case of AG-BG plant-mediated interactions. In the context of the simultaneous effects of BG on AG and reciprocal effects of AG on BG herbivores, cross-talk remains unreported. Here I showed evidence of cross-talk in leaves and roots of tomato plants challenged with RKN in roots and caterpillar on leaves. At the RKN *M. incognita* galling stage, I found a positive effect of the RKN on the caterpillar performance. At the same stage, the JA levels were enhanced while SA levels decreased, suggesting JA-SA cross-talk (see JA levels in Chapter 2 and SA levels in Figure S1). In roots, caterpillar feeding on RKN-infected plants lowered the JA levels while SA levels remained enhanced when

the RKN was at the reproduction stage, suggesting SA-JA cross-talk in roots (Chapter 3). These results indicate that caterpillar feeding on RKN infected plants trigger negative cross-talk between the JA and SA pathways in leaves during early RKN root infection, and in roots during the reproduction stage of the RKN. In the case of aphid feeding on RKN infected plants, there was no interference with the phytohormonal pathways either locally or systemically. Based on the experiment in these studies, it was not possible to discern the ecological role of the JA-SA pathways cross-talk under RKN-tomato-caterpillar interaction. Overall, these results demonstrate that cross-talk occurring in plants challenged by RKN and AG chewing herbivores is modulated by the nematodes' infection cycle.

The biosynthesis of secondary metabolites in response to herbivory is widespread in the plant kingdom. Here I used a metabolomics approach to decipher whether changes in the steroidal glycoalkaloid (SGA) *α*-tomatine and the steroidal glycoalkaloid metabolism (GAME) genes would correlate with observed insect's performances.

I found that root infection by the RKN increased *α*-tomatine in roots but not systemically in leaves (Chapters 2-4, and Figure S2). The induction of *α*-tomatine in roots was strongest when the RKN was at the galling and reproduction stages. The expression of GAME genes was not altered in leaves but was differentially affected in roots. I found no effect on the expression of GAME genes at the invasion and reproduction stages, but they were upregulated at the galling stage (Chapter 4). In line with these results, a previous report showed the increased concentration of *α*-tomatine in tomato roots after infection by the RKN *M. incognita* (Elliger et al., 1988). The SGAs are among the major allelochemicals in tomato plants that confer resistance towards many types of BG and AG herbivores (Elliger et al., 1988; Friedman, 2004; Wuyts et al., 2006; Ökmen et al., 2013; Garcia et al., 2018). Despite increased levels of *α*-tomatine in roots, I found no evident negative effects on the performance of the RKN *M. incognita*; I counted a high number of root galls at both the galling and reproduction stages. These results corroborate with another study that found high infection levels of RKN *M. incognita* in tomato plants with high *α*-tomatine levels (Elliger et al., 1988). The ability of parasitic root nematodes to secrete effector proteins that can neutralize the strength of induced defenses helps them to survive in a defended host (Haegeman et al., 2012; Grossi-de-Sa et al., 2019). Indeed, based on the present literature on nematode effector proteins, it can be deduced that the RKN *M. incognita* secretes effector proteins that are developmentally regulated to meet the needs of each life cycle stage. For example, studies using *N. benthamiana*, *A. thaliana*, and *Lycopersicon esculentum* to express effector proteins of the RKN *M. incognita* showed that the effectors *MiISE5, MiISE6, Mi-CM3* are secreted during early infections to facilitate the establishment of feeding sites (Shi et al., 2018b, 2018a; Wang et al., 2018). Moreover, the RKN *M. incognita* secretes the effector *Misp12* during the late infection stages to help in maintaining the giant cells and reproduction (Xie et al., 2016). The effector proteins mentioned above interfere with hormonal pathways and dependent responses, highlighting the possibility

also to affect secondary metabolites. Collectively, the utilization of effector protein to interfere with plant defense response can explain the lack of induction on *α*-tomatine at the early root infection stage and the lack of negative effects on RKN development.

Leaf feeding by caterpillar neither affected the levels of *α*-tomatine locally in leaves nor systemically in roots (Chapters 2,3). In contrast, the caterpillar downregulated the GAME genes in the roots of the flowering plants (Chapter 3). The aphid reduced the levels of *α*-tomatine and downregulated the GAME genes locally in leaves of young plants but did not trigger systemic effects on the SGA levels and expression of GAME genes in roots (Chapter 4). AG insect herbivores can overcome or suppress induced defenses. Caterpillars and aphids saliva secretions contain molecules that help to reduce the harmful effects of induced defenses. For instance, oral secretions of *S. exigua* contain glucose oxidase that elicits an SA burst potentially to attenuate the JA and dependent responses, as demonstrated in *N. attentuata* (Diezel et al., 2009). Aphids secrete effector proteins, for instance, *Me47* in the case of *M. euphorbiae*, suppress tomato defense responses and enhances the aphid's fecundity (Kettles and Kaloshian, 2016). Combining these studies and the fact these herbivores triggered phytohormonal responses that did not result in the production of SGAs, I suggest that these insect herbivores manipulate the phytohormone pathways by blocking important components for production metabolites. This, in return, might underlie the decrease in *α*-tomatine levels, the downregulation of the expression of GAME genes, and even the lack of effects reported in this thesis.

The activation of signaling pathways by an initial attacker can affect a second attacker via alterations in the secondary metabolites. I found that on RKN infected plants, feeding by the caterpillar and aphid slightly increased the levels of *α*-tomatine in leaves only when the nematode was at the invasion stage. In contrast, the expression of GAME genes in leaves was not altered by these insect herbivores when fed on RKN infected plants. In roots, leaf-feeding by caterpillar or aphid did not affect the RKN-induced *α*-tomatine levels or expression of GAME genes regardless of the nematodes' infection cycle stage. These results indicate that the AG insect herbivores do not overrule the effect induced on the GAME pathway by the RKN in the roots. Data on SGAs within the framework plant-mediated AG-BG herbivore interactions is un-available. Also, how the modulation of phytohormone pathways by the RKN might influence the production of SGAs is unknown. Here, the studies involving caterpillar feeding demonstrate that increase in JA levels after caterpillar leaf feeding on RKN infected plants corresponded with increased *α*-tomatine levels in leaves, at the nematodes' galling stage. Future work should identify and evaluate the physiological functions of SGAs on plant cells and signaling processes associated with the RKN plant interaction throughout their infection cycle, and how such effects can influence herbivores communities in AG.

Besides the induction of defense compounds, changes in nutrients can underlie the AG–BG plant-mediated interactions. Changes in plant nutritional quality upon AG and BG herbivory end up in an

asymmetrical interaction. For example, root-feeding herbivores damage the root system, and thus directly reduce the plant's ability to take up water and nutrients. Such damage on the root systems can propagate through the plant to the shoots, resulting in changes in the nutritional quality and influence AG herbivores. A model proposed by Masters and colleagues (1993) demonstrated that root-feeding herbivores cause drought-like stress, which influences the performance and fitness of shoot associated herbivores positively. In this case, changes in water content and nutrients uptake results in increased concentrations of free amino acids and soluble carbohydrates that benefit the herbivores (Masters et al., 1993; Jamieson et al., 2017). Based on these studies, I hypothesize that because the species of nematode that used in this thesis can modify the root structure, such modification might have interfered with the AG nutrient quality to affect the caterpillar positively especially in the galling stage. To investigate this, I measure the elemental carbon (C) and nitrogen (N) as their allocation patterns in tissues can indicate future utilization for compensatory growth or induction of defense to most vulnerable tissues (Creelman and Mullet, 1997; Wang et al., 2016; Kafle et al., 2017). I found that caterpillar feeding on RKN infected plants increased the C/N ratio in leaves, especially at the nematodes' galling stage. Whereas high levels of C/N ratio is an indicator of low-quality plants (Bryant et al., 1983; Luo et al., 2006; Dáder et al., 2016), I observed better performance of the caterpillar at the galling stage of the RKN. I speculate that by lowering the plant quality, less secondary metabolites might have been produced to defend the plant against the caterpillar. However, more studies are needed to test this thought.

**Linking induced plant traits under sequential root-shoot attack and to insect communities**

On both roots and shoots, plants interact with herbivores that can occur simultaneously and sequentially. Such interactions represent an intricate network that occurs at all levels of biological and ecological complexity (Cahill and Lamb, 2007; Stam et al., 2014). These interactions involve tens to hundreds of biotic species at the community level and are mainly mediated by hundreds to thousands of plant compounds and genes commonly regulated by hormonal networks (Hatcher et al., 2004; Erb et al., 2012; Checker et al., 2018). Each hormonal pathway has its own dynamics and the transcripts that result after herbivory influence the pathways that underlie the change in plant phenotype (Stam et al., 2014). The induced traits/phenotype underlie the plant's interaction with members of a particular community and consequentially can influence the community dynamics (Utsumi, 2013; Erb, 2018).

Linking the induced traits at different levels of ecological integration is a major challenge. However, studies investigating mechanisms underlying plant-mediated interactions can act as "building blocks" for the effects of such mechanisms at the community level. Community dynamics primarily results from a sequential attack on plants, where the first herbivore modifies physiological processes (e.g., primary, secondary metabolism, and transcriptional patterns) not only at the local sites but systemically, and

afterward affect the responding herbivore (second arriving herbivore) (Kaplan et al., 2008b, 2009, 2011; Erb et al., 2009; Kutyniok and Muller, 2012; Kumar et al., 2016; Arce et al., 2017; Wang et al., 2017; Kafle et al., 2017; Hoysted et al., 2018; Machado et al., 2018). In this thesis, chapters 2 and 4 agree with the studies mentioned above that root herbivores induce traits that can shape the AG insect communities. Moreover, using a RKN (always as the first inducer) and both caterpillar and aphids (responding herbivores), I showed that the RKN life cycle is a vital factor that underlies its effect on shoot insect herbivores and most likely at the community level. In this regard, I evaluate three models, i.e., priority effects, overriding effects, and canalization, to explain the effect of the RKN throughout the entire root infection cycle on AG insect herbivores, as well as the effect of AG herbivory on the induced root responses to the RKN.

Priority effects occur when the plant response depends on the sequence of arrival of insect herbivores on the host plant (Miller-Pierce and Preisser, 2012). They are predominant when the plant responses lead to an asymmetrical interaction. Asymmetrical plant-mediated interactions often occur and thus can be critical to shaping herbivore communities (Kaplan and Denno, 2007; Poelman et al., 2008; Miller-Pierce and Preisser, 2012). Competition between the interacting herbivores and cross-talk between induced responses are mechanisms suggested to drive priority effects (Kaplan et al., 2011; Hoysted et al., 2017; van Dam et al., 2018). Kaplan et al. (2011) found that root infection by the RKN *M. incognita* reduced aphid populations; these researchers attributed this effect to competition between the herbivores. Here I showed that root infection by RKN can differentially affect AG herbivores depending on the life history stages of the nematode feeding on the same plant. For example, caterpillars feeding on RKN infected tomato plants performed better only when feeding on plants in which the RKN was at the galling stage (Chapter 2). Hormonal cross-talk is well recognized and can underlie priority effects (Thaler et al., 2002, 2012; Pieterse et al., 2009). Here, AG feeding on RKN infected plants triggered JA-SA cross-talk in leaves at different stages of the nematode infection cycle: caterpillar at invasion and galling stages (see JA levels in Chapter 2 and SA levels in Figure S1) while aphids feeding did not cause cross-talk between phytohormones (Chapter 4). Considering that the aphids were not affected by RKN in any life stage, my combined results suggest that in asymmetrical interactions, priority effects may depend on the herbivores' feeding style, specialization, and the system under investigation.

Overriding effects occur when the later-arriving or responding herbivore overrules the induced effects by the first herbivore while sharing a host plant (van Zandt and Agrawal, 2004; Erb et al., 2011). I showed that caterpillar feeding on RKN infected plants suppressed the JA levels in roots, mainly when the nematode was at the reproduction stage. The aphids did not affect any of the RKN induced hormonal responses in roots. These results suggest that the overriding effects of the AG herbivore on the RKN induced responses is modulated by the nematodes' infection cycle and the type of the AG insect herbivore.

Canalization occurs when the first herbivore alone determines the plant's response, regardless of responding herbivores (Viswanathan et al., 2007; Stam et al., 2014). This effect reduces the plant's ability to be flexible in its response to other herbivores and consequently may affect the development and composition of the herbivore community. For example, I reported that root infection by RKN (first inducer) increased SA levels in both roots and leaves, which did not change even when the plants were exposed to the aphid later. This means that RKN can canalize root and leaf phytohormone responses. The increase in root SA levels has been shown to enhance resistance in tomato plants to RKN (Molinari et al., 2014). Here, I counted a high number of root galls at the galling and reproduction stages of the RKN, indicating successful infection and development. However, it is not possible to conclude on the effect of canalization on the performance of the RKN, because the feeding by the AG insect herbivore may have been for a short time. More investigations are needed to provide more details and evidence on the effect of canalization on RKN performance throughout its life cycle.

**Conclusions and prospects for further research**

The study findings presented in this thesis are useful insights in the framework of combined molecular and chemical analysis to elucidate plant physiology and the dynamics of AG-BG plant-mediated interactions. An eco-genomic approach at different organization levels was employed to provide insights on how plants mediate the interaction between RKN throughout its life cycle and AG insect herbivores. These studies are just the starting point and open up an exciting line of research to consider the life cycle of parasitic nematodes as an important factor that contributes to the outcome of AG-BG plant-mediated interactions. Some exciting prospects for future research would be to investigate the role of specific phytohormones using hormonal impaired mutants or transgenic/edited lines. Moreover, restraining the hormonal impairment to shoot or root organs by the use of grafting would provide essential information on the specific role played by each hormone and in each organ, and the central signaling involved in the AG-BG interactions. Another potential prospect would be to elucidate the impact of the RKN life cycle on the induction of indirect defenses, including the release of volatiles and the attraction of natural enemies. This would be important, especially in cases where the host plant shows increased susceptibility to herbivory. Considering the anticipated drastic climatic changes, it is also imperative to investigate how plants mediate the interaction between RKN and AG insect herbivores under fluctuating abiotic conditions. For example, under different temperature regimes, drought, or flooding. With that, I hope that the insights provided in this thesis will benefit some of the ongoing and future studies, especially those aiming to understand the plant-mediated interactions between parasitic root nematodes and AG insect herbivores beyond the second trophic level

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## SUPPORTING INFORMATION

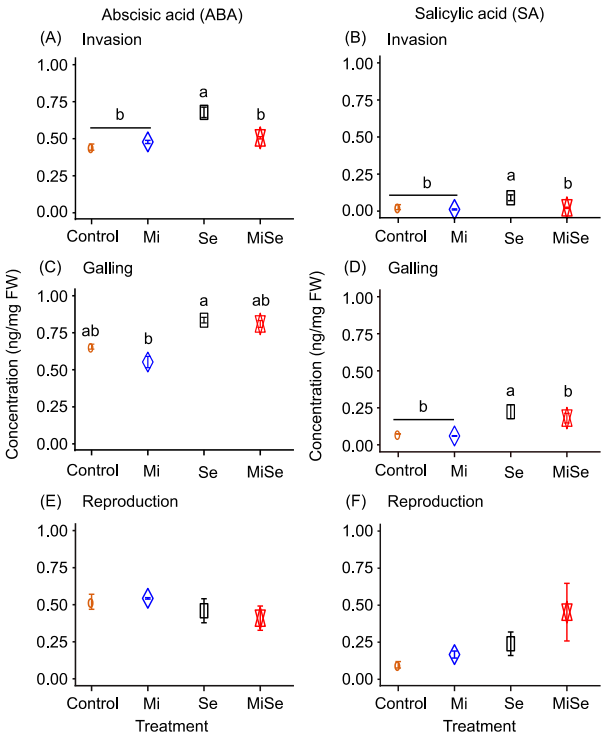


**Figure S1 Leaf phytohormone concentrations in tomato plants upon below and aboveground herbivory.** Concentrations of ABA (A,C,E), and SA (B,D,F) were measured in leaves of tomato plants not infested (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A,B), galling (C,D) or reproduction (E,F) stages. Samples were taken 24 hours after infestation with *S. exigua*. Data are the mean ± standard error (n=5-10). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

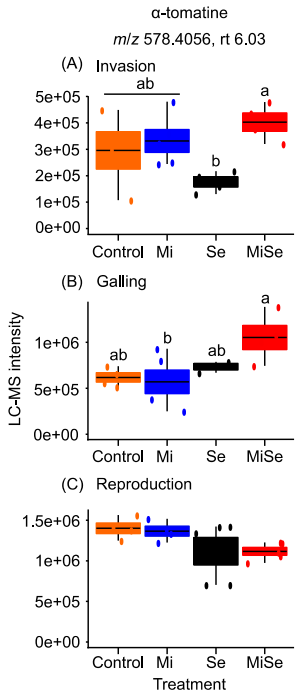


**Figure S2 LC-MS intensity of the steroidal glycoalkaloid α-tomatine in tomato leaves upon below and aboveground herbivory.** LC-MS intensity and mass to charge ratio (*m*/*z*) and retention time (rt) α-tomatine (A,B,C) measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion (A), galling (B), and reproduction (C) stages. Samples were taken 24 hours after infestation with *S. exigua*. Data are the mean ± standard error (*n*=5). Different letters indicate significant differences between treatments, determined by Tukeys HSD test for multiple comparisons after Two-Way ANOVA at $P \leq 0.05$.

**Table S1 ANOVA results on the concentrations of phytohormones in tomato leaves upon below and aboveground herbivory**

Concentrations of abscisic acid (ABA) and salicylic acid (SA) were measured in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi) or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after infestation with *S. exigua*. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Phytohormone | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Df | F | P | Df | F | P | Df | F | P |
| ABA | Mi | 1,27 | 10.436 | **0.003** | 1,13 | 6.0747 | **0.028** | 1,16 | 0.0456 | 0.834 |
| | Se | 1,27 | 43.994 | **<0.000** | 1,13 | 80.4095 | **<0.000** | 1,16 | 2.3664 | 0.144 |
| | MiSe | 1,27 | 19.772 | **0.000** | 1,13 | 3.6685 | 0.078 | 1,16 | 0.3385 | 0.569 |
| SA | Mi | 1,26 | 11.3872 | **0.023** | 1,12 | 0.7664 | 0.399 | 1,15 | 1.6700 | 0.216 |
| | Se | 1,26 | 22.1075 | **<0.000** | 1,12 | 12.7078 | **0.004** | 1,15 | 3.4739 | 0.082 |
| | MiSe | 1,26 | 9.0112 | **0.006** | 1,12 | 0.1449 | 0.710 | 1,15 | 0.4075 | 0.533 |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

**Table S2 ANOVA results on the LC-MS intensity of α-tomatine in tomato leaves upon below and aboveground herbivory.** The LC-MS intensity α-tomatine were determined in leaves of tomato plants without herbivores (Control), infected with *Meloidogyne incognita* (Mi), or *Spodoptera exigua* (Se) alone, or double infected with both herbivores (MiSe). In double infected plants, infestation with *S. exigua* was performed either at the nematodes' invasion, galling, or reproduction stages. Samples were taken 24 hours after infestation with *S. exigua*. Data were analyzed using a Two-way ANOVA linear model consisting of *M. incognita* (Mi), *S. exigua* (Se), and their interaction (Mi*Se) as model explanatory factors. The differences between the treatments were detected by Tukeys HSD test for multiple comparisons at $P \leq 0.05$. Statistically significant effects are indicated in bold.

| Mass to charge ratio (*m*/*z*) and retention time (rt) | Predicted identity | Source of variation | Invasion | | | Galling | | | Reproduction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Df (n,d) | F | P | Df (n,d) | F | P | Df (n,d) | F | P |
| 578.4050, 6.03 | α-tomatine | Mi | 1,13 | 7.5240 | **0.017** | 1,12 | 1.0117 | 0.334 | 1,14 | 0.0245 | 0.878 |
| | | Se | 1,13 | 0.1717 | 0.685 | 1,12 | 8.5875 | **0.013** | 1,14 | 6.1161 | **0.027** |
| | | MiSe | 1,13 | 4.2871 | 0.059 | 1,12 | 2.6976 | 0.126 | 1,14 | 0.0237 | 0.880 |

§; Df: degree of freedom, (n,d): numerator and denominator of Df, F: F-statistics value, P: probability value.

# ANNEX

Annex 1

# Leaf herbivory by *Manduca sexta* impairs *Meloidogyne incognita* root infection by counteracting the nematode's ability to manipulate root defenses *via* shoot-to-root jasmonate signaling

Ainhoa Martinez-Medina[1,2], Crispus M. Mbaluto[1,2], Anne Maedicke[1,2], Alexander Weinhold[1,2]; Fredd Vergara[1] and Nicole M van Dam[1,2].

[1]Molecular Interaction Ecology, German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany. [2]Institute of Biodiversity, Friedrich Schiller University Jena, Dornburger Str. 159, 07743 Jena. Germany

**Manuscript under preparation**

**Summary**

- Shoot herbivores may influence communities of herbivores associated with the roots *via* inducible defenses. However, the molecular mechanisms and hormonal signaling underpinning systemic impacts of leaf herbivory on root-induced responses against nematodes remain unclear.
- By using tomato as a model plant, we explored the impact of leaf herbivory by *Manduca sexta* on the performance of the root-knot nematode *Meloidogyne incognita*. By performing a sequence of glasshouse bioassays, we found that leaf herbivory delayed nematode root invasion and impaired nematode galling and reproduction. To explore the main molecular mechanisms involved in the shoot-to-root signaling involved, we performed bioassays with grafted plants compromised in jasmonate synthesis or perception, specifically in their shoots.
- We demonstrated the importance of an intact shoot jasmonate perception, but the jasmonate biosynthesis pathway was not essential in this shoot-to-root interaction. By analyzing the root expression profile of a set of oxylipin-related marker genes and untargeted metabolomics, we show that leaf herbivory counteracts the ability of *M. incognita* to downregulate jasmonates-related root defenses.
- Our results highlight the impact of leaf herbivory on the ability of *M. incognita* to manipulate root defenses and point to an important role of the jasmonate signaling pathway in the shoot-to-root signaling.

**Keywords**: Aboveground-belowground interactions; herbivory; *Manduca sexta*; metabolomics; plant resistance; root knot nematodes; shoot-to-root signaling; tomato

## INTRODUCTION

Plants are constantly subjected to a range of detrimental organisms that attack above and belowground (BG) plant parts. To prevent consumption by insect herbivores, plants can activate their defense arsenal upon recognition of the attacker encountered (Pieterse *et al*., 2009). Plant antiherbivore defense responses include, among others, the production of defensive proteins and toxic secondary metabolites that influence the herbivore's preference, feeding rate and/or development (Erb & Reymond, 2019). Herbivore-induced defense responses are regulated by a network of interconnected signaling pathways in which plant hormones play a major regulatory role (Erb & Reymond, 2019). Among them, the jasmonates (JAs), a family of oxylipins, emerged as key signals in plant responses to insect chewing insects herbivores such as beetles and caterpillars (Howe & Jander, 2008). Moreover, other hormones, such as salicylic acid, abscisic acid (ABA), ethylene, and auxins (AUX) may interact with the jasmonate-regulated signaling pathway in the orchestration of plant defenses against herbivores (Erb *et al*., 2012; Machado *et al*., 2016). Herbivore induced defenses are typically expressed not only locally at the damaged tissue but also systemically in undamaged plant parts that are spatially separated from the first inducer (Heil & Ton, 2008). Such a systemic response enables plants to protect the still undamaged tissues from herbivory and can influence the performance of other organisms that are feeding on the same plant, either simultaneously or later (Karban & Baldwin 1997; Soler *et al*., 2007, 2008). As a consequence, plants facilitate interactions between herbivorous insects that rarely come into direct physical contact with one another (Bezemer & van Dam, 2005; Soler *et al*., 2013).

The majority of studies on plant-mediated interactions between herbivores were constrained to aboveground (AG) tissues. However, a growing body of evidence shows that plant-mediated interactions *via* changes in inducible defenses also occur between AG and BG organisms (Papadopoulou & van Dam, 2017). For instance, BG herbivory can increase the level of plant defense compounds, such as terpenoids, glucosinolates or phenolics in AG plant tissues affecting herbivorous species feeding AG of the same plants (Bezemer *et al*., 2003, 2004; van Dam, *et al*., 2004, 2005; Hol *et al*., 2004). Defensive properties of the roots have been less studied compared to AG plant parts. However, few studies show that AG herbivory can also induce defenses systemically in BG tissues, affecting plant interaction with root-feeding organisms (Bezemer *et al*., 2004; Soler *et al*., 2007; Erb *et al*., 2015; Machado *et al*., 2018). Several compounds, such as JAs, ABA, and AUX, play important roles in AG-BG signaling (Erb *et al*., 2009; Machado *et al*., 2013; Fragoso *et al*., 2014; Schulze *et al*., 2019). However, the mechanisms driving these systemic effects and the long-distance signals involved remain poorly understood. More specifically, very little information is available about the molecular mechanisms and signaling underlying the systemic impact of leaf herbivory on root defensive responses against plant parasites as root-knot nematodes (RKN).

Root-knot nematodes are parasitic animals able to manipulate plant defenses and reprogram feeding cells in the roots in order to supply themselves with nutrients (Gheysen & Mitchum, 2011). The infective second-stage juveniles penetrate the host root near the zone of elongation and migrate intercellularly towards the vascular cylinder, where they establish feeding sites, known as giant cells. Hyperplasia and hypertrophy of the surrounding cells lead to the formation of macroscopically visible root knots or galls in which the nematodes are embedded (Kyndt *et al*., 2014). As obligate endoparasites that complete most of their life cycle inside plant roots, the ability of RKNs to maintain their feeding sites rely on continuous modulation of plant defenses (Goverse & Smant, 2014). Several signaling molecules are involved in the plant defense responses mounted against RKNs. Among them, jasmonates play a major role in basal and induced defenses against RKNs in several plant species (Cooper *et al*., 2005; Nahar *et al*., 2011; Fujimoto *et al*., 2011; Gleason *et al*., 2016; Hu *et al*., 2017; Kyndt *et al*., 2017; Yimer *et al*., 2018).

Several studies demonstrate that foliar treatment with jasmonic acid or methyl jasmonate reduces the plant's susceptibility to RKNs BG, indicating the involvement of JAs in the shoot-to-root communication underlying the systemic protection against RKNs (Cooper *et al*., 2005; Nahar *et al*., 2011; Vieira dos Santos *et al*., 2013; Fujimoto *et al*., 2011; Fan *et al*., 2015). However, the specific mechanisms responsible for this phenomenon remain ambiguous. Moreover, studies addressing the impact of AG elicitation by shoot herbivory on RKNs infection are scarce and show conflicting results. For instance, transient shoot herbivory by the chewing herbivore *Spodoptera exigua* triggered a decrease in jasmonic acid levels in tomato roots and did not affect the number of galls induced by the RKN *Meloidogyne incognita* (Kafle *et al*., 2017). By contrast, simulated herbivory by *Manduca sexta* larvae strongly induced JAs in the root of tobacco plants and led to an increase in the number of *M. incognita* eggs (Machado *et al*., 2018).

The long-term root interaction with RKN is highly complex and dynamic. The outcome of the interaction between nematodes and the plant results from the continuous interplay between the active manipulation of host defenses by nematode effectors secreted in the plant tissue to promote susceptibility, and defense responses triggered by the plant to control the infection (Goverse & Bird, 2011; Goverse & Smant, 2014; Ibrahim *et al*., 2019). Accordingly, we hypothesized that systemic elicitation of root defenses by leaf herbivory counteracts the ability of the RKN *M. incognita* to manipulate root defenses to its own benefit, thereby negatively affecting its infection success. By performing a sequence of glasshouse bioassays, we found that continuous leaf herbivory by the caterpillar *M. sexta* delayed nematode root invasion and negatively affected nematodes' galling and reproduction. To understand the main molecular mechanisms involved in the shoot-to-root signaling, we performed bioassays with grafted plants compromised in JAs synthesis or signaling in their shoots. This demonstrated the importance of an intact shoot jasmonate signaling, whereas *de novo* shoot jasmonate biosynthesis was not required to enhance

resistance against *M. incognita*. By analyzing the expression profile of a set of oxylipin-related genes combined with untargeted metabolomics, we showed that *M. sexta* leaf herbivory counteracts the ability of *M. incognita* to downregulate jasmonate-related root defenses. Our results highlight the impact of leaf herbivory on the ability of the RKN *M. incognita* to manipulate root defenses to its benefit and point to an important role for jasmonate signaling in shoot-to-root signaling.

## MATERIAL AND METHODS

### Plant, nematode and insect material

We used tomato (*Solanum lycopersicum*) cultivar 'Moneymaker' in the bioassays unless indicated otherwise. Also, we used the tomato mutant lines *spr2* (*suppressor of prosystemin-mediated responses2*; Li *et al*., 2003) and *def1* (*defenseless-1*; Howe *et al*., 1996) compromised in jasmonates biosynthesis; and the mutant line *jai1* (*jasmonic acid-insensitive1*; Li *et al*., 2004) compromised in jasmonates signaling, all in background Castelmart. Seeds were kindly provided by Prof. Pozo (EEZ-CSIC). We germinated the seeds from the background Castlemart and the lines *spr2* and *def1* for 10 days, as described by Martinez-Medina *et al*. (2017). The seeds from the line *jai1* were germinated on a water-saturated filter paper in the dark at 25 °C, according to Li *et al*. (2004). We selected homozygous *jai1-1* seedlings from F2 populations, according to Li *et al*. (2004). The inoculum of the RKN *M. incognita* was produced, according to Martinez-Medina *et al*. (2017). We counted and adjusted *M. incognita* eggs to a suspension of 3000 eggs $ml^{-1}$ water, according to Martinez-Medina *et al*. (2017). *Manduca sexta* (Lepidoptera, Sphingidae) eggs were obtained from the Max Planck Institute for Chemical Ecology (Jena, Germany). The *M. sexta* culture was maintained, according to Grosse-Wilde *et al*. (2011).

### Plant growth and experimental design

We transplanted 10-day-old tomato seedlings in 400-ml pots filled with a sterile soil-sand mixture (12: 5, v: v) according to Martinez-Medina *et al*. (2017). We placed the plants in a glasshouse compartment under conditions of 25 ± 3 °C, 16-h light: 8-h dark, and 70 % relative humidity. Plants were watered three times a week, alternately with tap water and half-strength Hoagland solution (Hoagland & Arnon, 1938). After 3 weeks, we used the plants for the experiments. We used a full factorial design, with the factors (*i*) root challenge with *M. incognita* and (*ii*) shoot challenge with *M. sexta*. For *M. incognita* treatments, we inoculated the plants with ~3000 fresh eggs of *M. incognita* per root by injecting 1 ml of an egg suspension (3000 eggs $ml^{-1}$) into the soil according to Martinez-Medina *et al*. (2017). Plants that were not assigned for nematode inoculation were mock-inoculated with 1 ml water. For *M. sexta* treatments 3 neonates were placed on the third fully-expanded leaf (counted from below) and allowed to feed freely on the entire plant. We replaced *M. sexta* larvae weekly with new neonates to avoid the consumption of the entire shoot

biomass. To assess the impact of *M. sexta* leaf herbivory on *M. incognita* root infection, plants were inoculated with *M. incognita* eggs and challenged at the same time with the *M. sexta* neonates. The bioassay consisted of four treatments: (1) control plants not challenged with any of the herbivores; (2) plants root-infected with *M. incognita*; (3) plants exposed to shoot-feeding by *M. sexta,* and (4) plants root-infected with *M. incognita* and exposed to *M. sexta* at the shoot. Ten biological replicates of each treatment per time-point were used, unless indicated otherwise. At 3, 7, and 21 days after the start of the experiment, the caterpillars were removed and the plants were immediately harvested. Root and shoot material was collected and stored at -80°C.

To assess the impact of *M. sexta* leaf herbivory on every specific stage of *M. incognita* infection, we performed three additional bioassays in the glasshouse, in which we varied the specific timing of shoot and root challenge (Fig.1). For assessing the impact of *M. sexta* leaf herbivory on *M. incognita* root invasion, we placed the *M. sexta* larvae on the shoot of the plants, and 12 hours later, we inoculated the roots with *M. incognita* (Fig.1a). At 3 and 7 days after *M. incognita* inoculation, roots were harvested and stored at -80 °C for the quantification of *M. incognita* DNA. For assessing the impact of *M. sexta* leaf herbivory on *M. incognita* galling, we first inoculated the plants with *M. incognita*, and one week later, after *M. incognita* had successfully invaded the roots, we challenged the plants with *M. sexta* larvae (Fig. 1b). Two weeks after challenging the plants with *M. sexta* (3 weeks after challenging the plants with *M. incognita*), we harvested the plants and assessed the number of roots galls. To study the impact of *M. sexta* leaf herbivory on *M. incognita* fecundity, we first inoculated the plants with *M. incognita*, and 3 weeks later (when *M. incognita* had successfully invaded the roots and developed inside) we challenged the plants with *M. sexta* (Fig.1c). Two weeks after challenging the plants with *M. sexta* (5 weeks after challenging the plants with *M. incognita*), we evaluated nematode fecundity.

**Tomato grafts**

Seeds from the wild type (wt) Castlemart and the jasmonate-compromised lines *spr2*, *def1*, and *jai1* were germinated and growth as described above. Three weeks after transplanting, we grafted scions of the wild type Castlemart and from the lines *spr2*, *def1*, and *jai1* onto rootstocks of the wild type Castlemart. Grafts were made by cutting the scion and rootstock plants diagonally (approx. 2 mm above the cotyledon) and securing the junction with a silicone clamp. Grafted plants were placed under 9-h light, 21°C : 15-h dark, 18°C, 90% relative humidity conditions. One week after grafting, the plants were used in the bioassays.

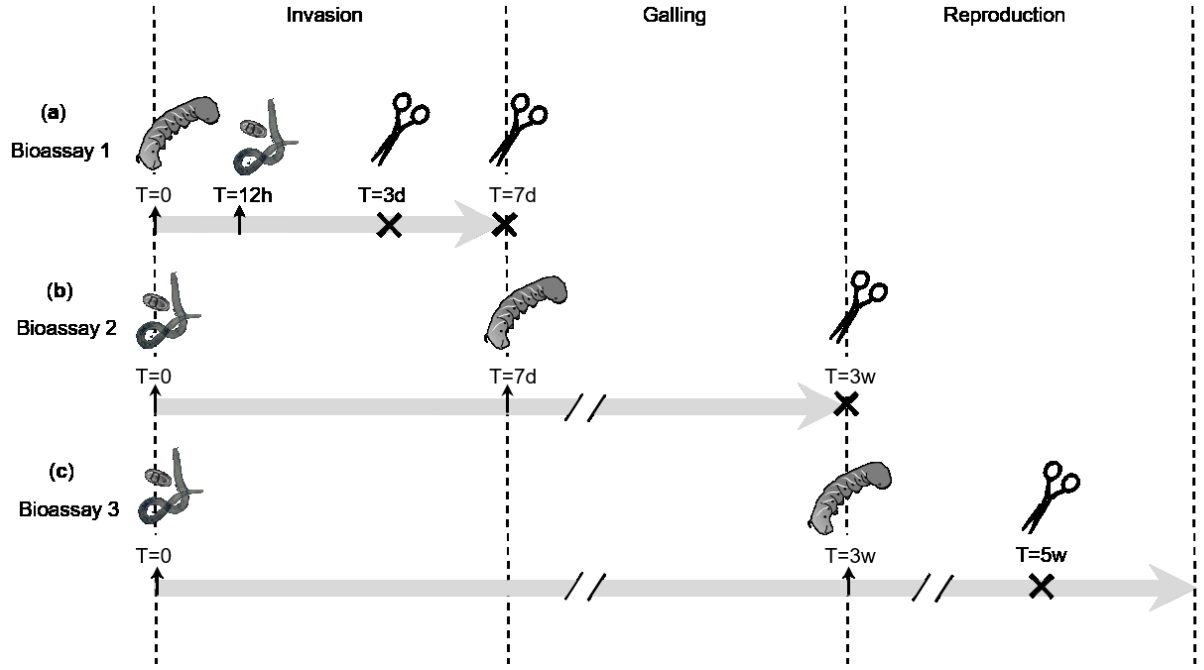


**Figure 1. Experimental study design to evaluate the impact of *Manduca sexta* leaf herbivory on specific stages of the *Meloidogyne incognita* infection cycle.** (a) To assess the impact of *M. sexta* leaf herbivory on *M. incognita* root invasion, plants were challenged with *M. sexta* and 12 hours later inoculated with *M. incognita* eggs. Roots were sampled 3 and 7 days after *M. incognita* inoculation. (b) To analyse the impact of *M. sexta* leaf herbivory on *M. incognita* galling, roots were inoculated with *M. incognita* eggs and 7 days later plants were challenged with *M. sexta.* Roots were sampled 3 weeks after *M. incognita* inoculation. (b) To evaluate the impact of *M. sexta* leaf herbivory on *M. incognita* reproduction, roots were inoculated with *M. incognita* eggs and 3 weeks later plants were challenged with *M. sexta*. Roots were sampled 5 weeks after *M. incognita* inoculation.

**Assessment of nematode behavior**

Root systems were carefully washed with tap water. To assess the impact of *M. sexta* shoot herbivory on *M. incognita* root invasion, we estimated *M. incognita* biomass by quantitative RT-PCR (qPCR) and the primers of the *Actin* gene from *M. incognita* according to Martinez-Medina *et al*. (2017). Nematode performance was analyzed by counting gall numbers on roots. Fecundity was determined by counting the number of egg clusters and the number of eggs per egg cluster, according to Martinez-Medina *et al*. (2017).

**Real-time quantitative RT-PCR**

For the determination of *M. incognita* biomass, the total DNA of roots of nematode infected tomato plants was extracted by using the DNeasy plant kit (Qiagen) according to the manufacturer's instructions. For gene expression analyses, we isolated total RNA of roots of tomato plants as described by Oñate-Sánchez & Vicente-Carbajosa (2008). We synthesized the first-strand cDNA from 1 µg DNase free RNA using Revert

Aid H-minus RT (Thermo Scientific) following the manufacturer's instructions. We performed real-time quantitative RT-PCR reactions according to Papadopoulou *et al.* (2018), and by using the gene-specific primers described in Supporting Information Table S1. For gene expression analysis, the data were normalized by using the housekeeping gene *SlEF* (X14449), encoding the tomato translation elongation factor-1α (Miranda *et al*., 2013). *M. incognita* DNA was estimated by analyzing *M. incognita Actin* gen (MINC06773a) relative to the *SlEF* gene.

**Phytohormone extraction and analysis**

We extracted root phytohormones from 100 mg of homogenous fresh root material, according to Escobar-Bravo *et al*. (2019), using ethyl acetate containing the internal standards (40 ng D6-SA, 40 ng D6-ABA, 40 ng d5-IAA, 40 ng D6-JA and 40 ng d6-JA-Ile) as the solvent. Data acquisition and processing were performed, according to Escobar-Bravo *et al*. (2019). Phytohormone levels were calculated over the amount of fresh mass of plant material ($ng^{-1}$ $mg^{-1}$ fresh mass) and the peak values of the corresponding internal standards.

**Metabolites extraction and data processing**

We extracted 100 mg fresh root tissue of each sample in 1 ml of extraction buffer, as described in Methods S1. We performed chromatographic separation of all diluted extracts as described in Methods S1. A commercial standard of α-tomatine (Extrasynthese, Lyon, France) was injected with the same conditions, but the scan range was modified to 50–1500 *m*/*z*. Processing of the liquid chromatography-mass spectrometry data was performed as described in Methods S2. We interpreted the mass spectra of the most variable loadings and hypothesized structures when possible. We produced structural hypotheses based on characteristics like mass fragmentation, presence of inorganic adducts, and comparisons with previously reported mass spectra in MassBank of North America. We normalized the alignments against the total ion chromatogram. We used the ion relative intensity values of characteristics signals for each of our predicted structures for comparison of compound abundance in different treatments.

**Statistical analysis**

We analyzed all datasets by ANOVA using the software *R* (version 3.1.2). Normality and equality of variance were verified using Shapiro–Wilk and Levene's tests, respectively. Following two-way ANOVAs, one-way ANOVAs were performed for each time-point to analyze the impact of *M. sexta* leaf herbivory on *M. incognita*-triggered transcriptomic and metabolomic root alterations. Tukey's test was used for overall comparisons. When ANOVA assumptions were not met, Dunnett's test was performed to detect differences among treatments. Student's t-test was used for pairwise comparisons.

## RESULTS

### Shoot herbivory by *Manduca sexta* reduces *Meloidogyne incognita* performance by delaying invasion and impeding development and fecundity

We first studied whether continuous leaf herbivory by *M. sexta* impacts the performance of *M. incognita*. To this end, we challenged tomato plants with *M. incognita* alone or with both *M. sexta* and *M. incognita*, and 3 weeks later, we evaluated the number of root galls. Shoot herbivory by *M. sexta* led to a reduction (up to 50 %) in the number of root galls induced by *M. incognita* (Fig. 2), indicating that *M. sexta* leaf herbivory impairs *M. incognita* infection. By contrast, root infection by *M. incognita* did not affect *M. sexta* larval growth (Fig. S1). The root interaction with RKNs involves different stages, namely: invasion, development, and reproduction. Next, we aimed to identify the specific stages of the nematode infection life-cycle, which are affected by leaf herbivory. To this end, we performed 3 different bioassays in the greenhouse, in which we varied the specific timing of the shoot and root challenge (Fig.1). In the first bioassay, we assessed the impact of *M. sexta* leaf herbivory on *M. incognita* root invasion (Fig.1a). At 3 days after nematode inoculation, we found a decrease in *M. incognita* DNA in *M. incognita*-infected roots of plants that were also challenged with *M. sexta*, compared to roots of plants challenged with *M. incognita* alone (Fig. 3a). However, 7 days after nematode inoculation, *M. incognita* DNA levels were similar in roots of plants inoculated with *M. incognita* alone and plants challenged by both *M. incognita* and *M. sexta* (Fig. 3a). These results indicate that leaf herbivory by *M. sexta* leads to a delay in *M. incognita* root invasion. Next, we investigated the impact of leaf herbivory on nematode galling (Fig.1b). *M. sexta* leaf herbivory led to a reduction in the number of *M. incognita* root galls per root system (Fig. 3b), indicating that leaf herbivory impairs the development of the nematodes inside the root tissues. We finally studied whether *M. sexta* leaf herbivory affects *M. incognita* fecundity (Fig.1c). The percentage of egg clusters-containing galls and the number of eggs per egg cluster decreased in the roots of plants challenged with both *M. incognita* and *M. sexta*, compared to plants inoculated with *M. incognita* alone (Fig. 3c). Collectively, these results show that *M. sexta* leaf herbivory reduces *M. incognita* performance by delaying nematode root invasion and by impeding the development and the reproduction of nematodes inside the roots.

### Shoot jasmonates perception but not *de novo* synthesis is required for *Manduca sexta* systemic impairment of *Meloidogyne incognita* infection

We next studied whether the impact of *M. sexta* feeding on *M. incognita* root infection is mediated by jasmonate signaling. With this aim, we used grafted plants with compromised jasmonate synthesis or perception, specifically in their shoots (Fig. 4a). We first observed that in wt/wt grafted plants, *M. sexta* herbivory reduced the number of *M. incognita* root galls (Fig. 4b) in a similar manner to that observed in non-grafted plants (Fig. 2). Similarly, a reduction in the number of root galls by *M. sexta* was observed in

grafts composed by wt rootstock, and scions from the jasmonate-biosynthesis compromised lines *spr2* (compromised in wound-induced JA biosynthesis) or *def1* (with a defective octadecanoid synthesis pathway; Fig. 4b). We found, however, a contrasting result in grafts that included scions from the jasmonates-perception compromised line *jai1* (jasmonates insensitive 1; Fig. 4b). In *jai1*/wt grafts, *M. sexta* treatment failed in reducing the number of *M. incognita* root galls. Moreover, an increase in the number of root galls was observed in *M. sexta* challenged plants in *jai1*/wt grafts compared to non-*M. sexta* treatment (Fig. 4b). These observations indicate that *de novo* jasmonates biosynthesis in shoots is not required for *M. sexta*-triggered impairment of *M. incognita* root infection. However, an intact JAs perception seems to be required.

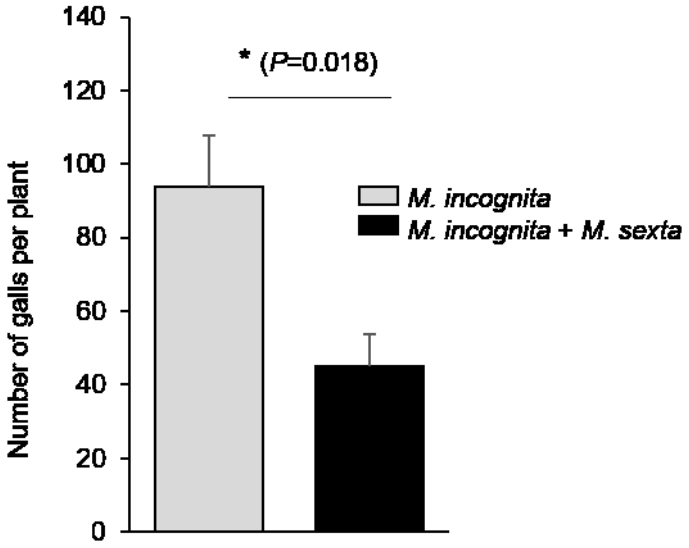


**Figure 2. *Manduca sexta* leaf herbivory impairs *Meloidogyne incognita* root infection.** Tomato plants were inoculated with *M. incognita* eggs and challenged or not with *M. sexta* larvae. Three weeks later the number of galls was counted. The bars indicate the average gall number (+SE). The asterisk indicates significant differences between the treatments according to Student's t-test ($P < 0.05$; n = 10).

### *Manduca sexta* leaf herbivory counteracts the ability of *Meloidogyne incognita* for suppressing the 13-LOX oxylipin pathway in tomato roots

Root-knot nematodes can modulate oxylipin-related root defenses to successfully parasitize their host (Gheysen & Mitchum, 2019). To understand whether leaf herbivory by *M. sexta* interferes with the ability of *M. incognita* to modulate the oxylipin pathway, we first studied the impact of *M. incognita* infection on the oxylipin pathway in tomato roots. In tomato, there are two major branches of the oxylipin pathway, the 13-LOX branch that leads to the family of jasmonates (Howe *et al*., 2018); and the 9-LOX branch (Itoh *et al*., 2002). We found a general transcriptional downregulation of the genes *LOXD* (*Lypoxygenase D*)*, AOS1*

(*Allene Oxide Synthase 1*), *AOS2* (*Allene Oxide Synthase 2*), *AOC* (*Allene Oxide Cyclase*), and *OPR3* (*12-Oxophytodienoic Acid Reductase 3*), encoding for key enzymes of the 13-LOX branch, in *M. incognita* infected roots (Fig. 5a, Table S2). This downregulation was observed specifically at 3 and 7 days after nematode inoculation, coinciding with the invasion and induction stages of *M. incognita* infection cycle. However, at 21 days after nematode inoculation, there were no significant differences in root expression of *LOXD*, *AOS1*, *AOS2*, *AOC*, and *OPR3* between *M. incognita* inoculated and control plants (Fig. 5a). In accordance, *M. incognita* root inoculation led to a general reduction of OPDA and JA levels in tomato roots at the early stages of nematode infection (Fig. 5b, Table S2, S3). In the case of OPDA, a reduction was observed in *M. incognita* infected roots 3 days after inoculation, while reduced levels of JA were observed both 3 and 7 days after inoculation. Root levels of OPDA and JA in *M. incognita* inoculated roots were similar to those found in control roots 21 days after *M. incognita* inoculation (Fig. 5b). *M. incognita* inoculation did not significantly affect JA-Ile (jasmonyl-*L*-isoleucine) levels in tomato roots, though a slight increase was observed 3 days after nematode inoculation (Fig. 5b). Noticeably, *M. incognita* root infection, in general, did not affect shoot levels of OPDA, JA nor JA-Ile (Fig S2). In contrast to the 13-LOX branch, *M. incognita* inoculation did not significantly affect the transcription levels of *LOXA* (*Lypoxygenase A*), *AOS3* (*Allene Oxide Synthase 3*) and *DES* (*Divinyl Ether Synthase*), encoding key enzymes of the 9-LOX branch (Fig. 6). Our results indicate that *M. incognita* infection led to an early and transient downregulation of the 13-LOX branch of the oxylipin pathway in tomato roots.

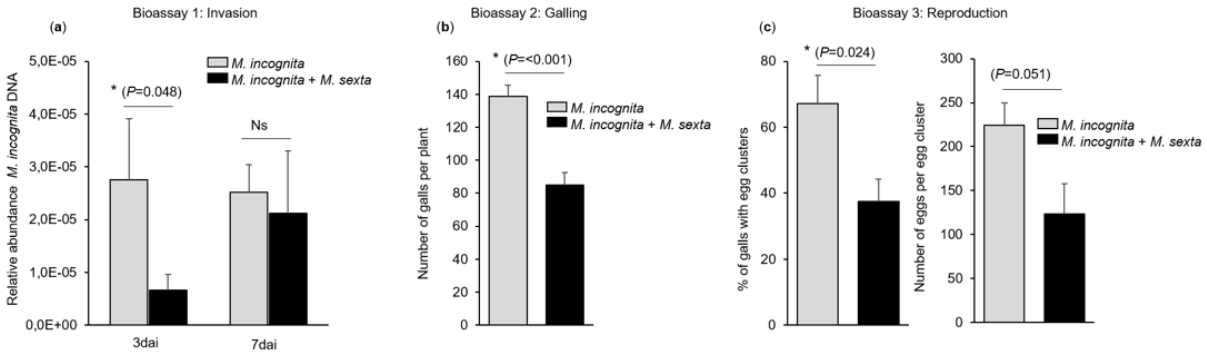


**Figure 3**. ***Manduca sexta*** **leaf herbivory affects** ***Meloidogyne incognita*** **infection cycle**. (a) The relative *M. incognita* DNA was measured in roots of plants root inoculated with *M. incognita* and challenged or not aboveground with *M. sexta.* Relative *M. incognita* DNA was estimated 3 and 7 days after *M. incognita* inoculation (dai) by analysing *M. incognita Actin* gen relative to *SlEF* gen. (b) The number of galls was quantified in roots inoculated with *M. incognita* and challenged or not aboveground with *M. sexta* 3 weeks after *M. incognita* inoculation. (c) Percentage of root galls showing egg clusters (left panel) and number of eggs per egg cluster (right panel) in the root of plants that were inoculated with *M. incognita* and challenged or not aboveground with *M. sexta*. Egg clusters were analysed and collected from tomato root tissue 5 weeks after *M. incognita* inoculation. Data are mean +SE (n = 10). The asterisk indicates significant differences between the treatments according to Student's t-test.

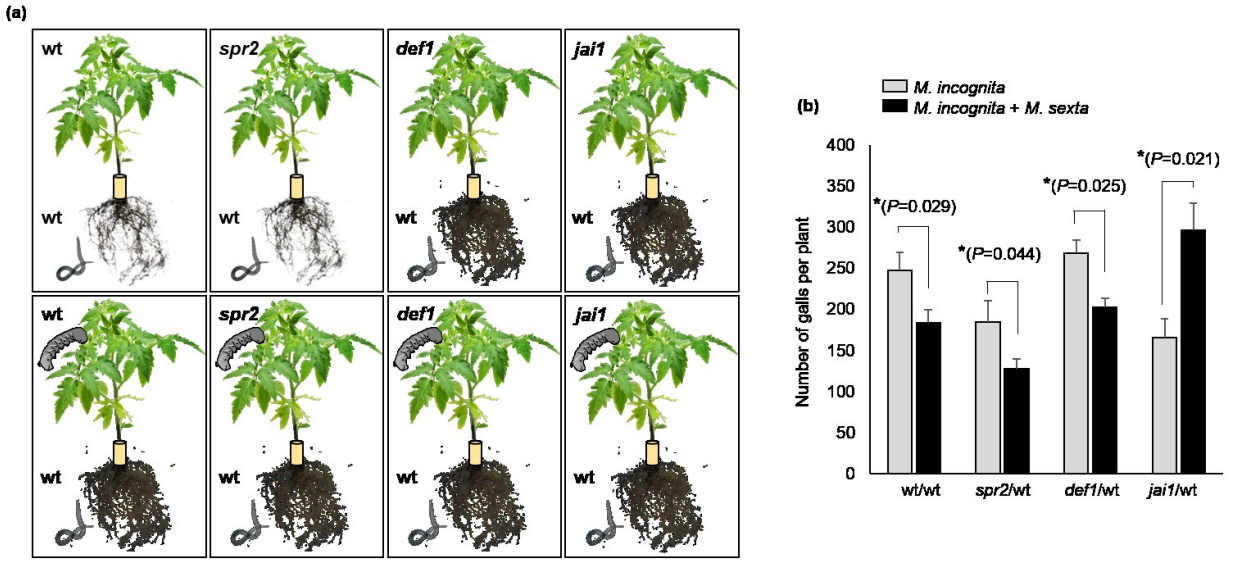


**Figure 4. The involvement of *de novo* shoot jasmonate synthesis and jasmonate perception in shoot-to-root *Manduca sexta-Meloidogyne incognita* interaction**. (a) grafts were made with rootstocks of the wild type Moneymaker and scions of the wild type Moneymaker (wt/wt), the jasmonate biosynthesis compromised lines *spr2 (spr2*/wt*)* and *def1* (*def1*/wt) or the jasmonate perception compromised line *jai1 (jai*/wt). One week after grafting, the plants were root inoculated with *M. incognita*. Half of the plants were also challenged aboveground with *M. sexta* (lower panels). (b) Three weeks after *M. incognita* inoculation the number of galls was evaluated. X-axis shows the graft combinations (scion/rootstock). The bars indicate average gall number (+SE). For each graft type, the asterisk indicates significant differences between the treatments according to Student's t-test ($P < 0.05$; n = 8)

We next aimed to study whether *M. sexta* leaf herbivory systemically affects *M. incognita* ability to downregulate the 13-LOX branch of the oxylipin pathway. *M. sexta* leaf herbivory triggered a general transcriptional activation of the 13-LOX branch marker genes LOXD, AOS1, AOS2, AOC, and OPR3 systemically in plant roots (Fig. 5a, Table S2). Shoot herbivory also systemically increased the levels of OPDA, JA, and JA-Ile in tomato roots, although this effect was time- and hormone-dependent (Fig. 5b, Table S2, S3). In the case of OPDA, an increase was observed in roots 3 days after shoot herbivory, while JA root levels were increased at 3 and 21 days after shoot herbivory (Fig. 5b). *M. sexta* herbivory led to a general increase in root JA-Ile levels, although this increase was statistically significant only after 21 days of herbivory (Fig. 5b). As expected, *M. sexta* herbivory also triggered an increase of OPDA, JA, and JA-Ile in tomato shoots (Fig. S2). Remarkably, in roots of plants that were challenged with both *M. sexta* and *M. incognita*, transcript levels of *LOXD*, *AOS1*, *AOS2*, *AOC*, and *OPR3* remained in general at a higher level compared to those found in the root of plants inoculated with *M. incognita* alone (Fig. 5a). Moreover, the expression pattern of *LOXD*, *AOS1*, *AOS2*, *AOC,* and *OPR3* in plants challenged with both *M. sexta* and *M. incognita* were more similar to the expression pattern found in plants challenged with *M. sexta*

alone, than to plants challenged with *M. incognita* alone (Fig. 5a). In accordance, the levels of OPDA and JA in plants challenged with both *M. sexta* and *M. incognita* remained in general higher compared to the levels observed in plants challenged with *M. incognita* alone (Fig. 5b). The levels of JA-Ile in plants challenged with *M. sexta* and *M. incognita* remained similar to the levels observed in control plants.

*M. sexta* leaf herbivory led to a systemic increase of the 9-LOX branch-marker genes *LOXA*, *AOS3,* and *DES* (Fig. 6, Table S2). *M. sexta* upregulated expression of *LOXA* in roots during the entire time of the experiment (Fig. 6a). By contrast, the impact of *M. sexta* on root expression of *AOS3* and *DES* was time-dependent (Fig. 6b,c). Root expression of *AOS3* was specifically upregulated at 3 days after *M. sexta* herbivory (Fig. 6b), while *DES* was upregulated at 7 and 21 days after herbivory (Fig. 6c). Remarkably, a similar expression pattern of *LOXA*, *AOS3,* and *DES* was found in plants challenged together with *M. sexta* plus *M. incognita* (Fig. 6). Our results show that *M. sexta* shoot herbivory systemically activates the 13-LOX and 9-LOX branches of the oxylipin pathway in tomato roots, and counteracts the *M. incognita*-triggered repression of the 13-LOX branch of the oxylipin pathway.

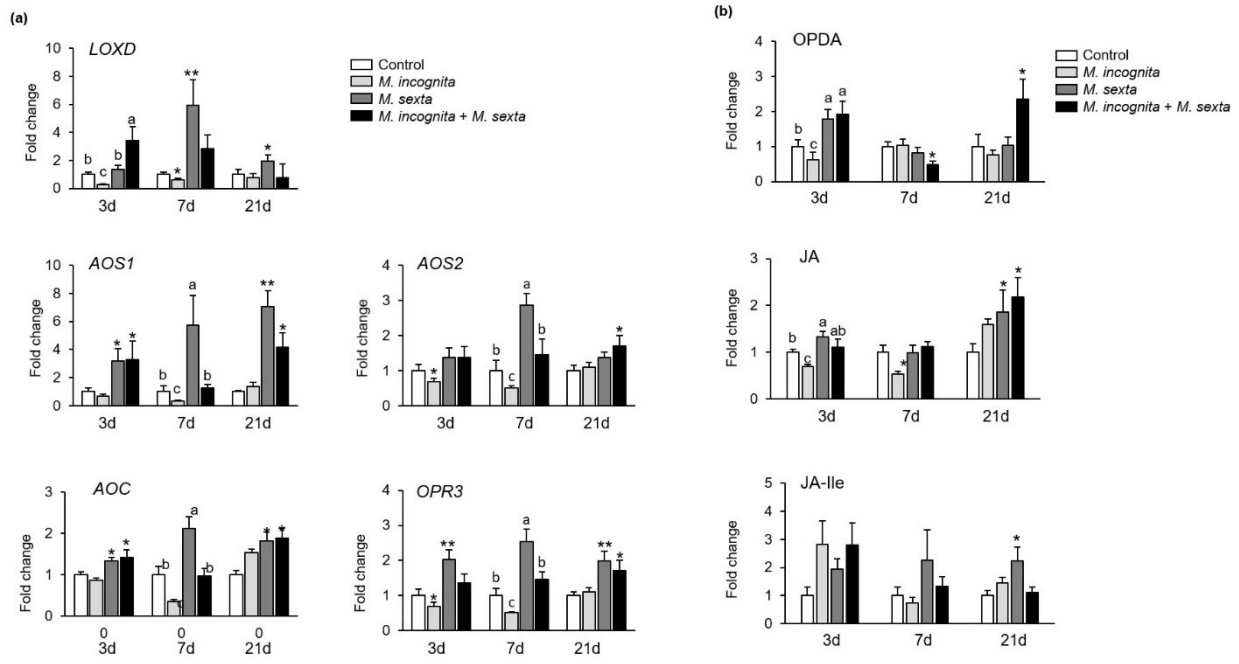


**Figure 5**. ***Manduca sexta* leaf herbivory antagonizes the repression of the 13-LOX oxylipin pathway triggered by *Meloidogyne incognita***. (a) Expression levels of the 13-LOX biosynthesis marker genes *LOXD* (*Lipoxygenase D*), *AOS1* (*Allene Oxide Synthase 1*), *AOS2* (*Allene Oxide Synthase 2*), *AOC* (*Allene Oxide Cyclase*), and *OPR3* (*12-Oxophytodienoic Acid Reductase 3)* and (b) root levels of OPDA (oxophytodienoic acid) JA (jasmonic acid) and JA-Ile (jasmonoyl-Isoleucine) were analysed in roots of plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Gene expression and metabolite contents were analysed 3, 7 and 21 days after *M. incognita*

inoculation. Data are mean + SE. In (a) the results are normalized to the *SlEF* gene expression levels, and expressed relative to those found in control plants at each sampling time, which were arbitrarily given a value of 1. In (b) the accumulation levels are expressed relative to those found in control plants at each sampling time, which were arbitrarily given a value of 1. At each time point different letters indicate differences between treatments according to Tukey's test following two-way ANOVA with factors *M. incognita* challenge and *M. sexta* challenge (Table S2). At each time point asterisks mean significantly different from control plants according to Dunnett's test ($P< 0.05$, n=5). *, $P < 0.05$; **, $P < 0.01$.

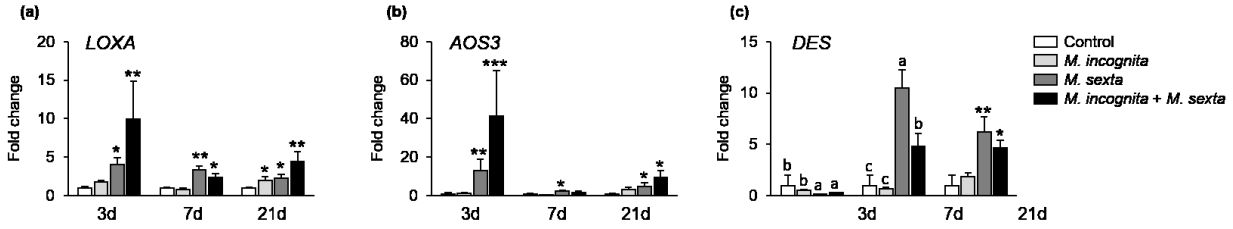


**Figure 6**. **Impact of *Meloidogyne incognita* and *Manduca sexta* on the 9-LOX oxylipin pathway in tomato roots**. Expression levels of the 9-LOX biosynthesis marker genes (a) *LOXA* (*Lipoxygenase A*), (b) *AOS3* (*Allene Oxide Synthase 3*) and (c) *DES* (*Divinyl Ether Synthase*) were analysed in roots of plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Gene expression was analysed at 3, 7 and 21 days after *M. incognita* inoculation. Data are mean+ SE. Results were normalized to the *SlEF* gene expression levels, and expressed relative to those found in control plants at each sampling time, which were arbitrarily given a value of 1. At each time point different letters indicate differences between treatments according to Tukey's test following two-way ANOVA with factors *M. incognita* challenge and *M. sexta* challenge (Table S2). At each time point asterisks mean significantly different from control plants according to Dunnett's test ($P< 0.05$, n=5). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

**Leaf herbivory by *Manduca sexta* alters systemically the root metabolomic signature triggered by *Meloidogyne incognita* infection**

We next investigated whether the systemic impact of *M. sexta* leaf herbivory on the root oxylipin pathway was associated with systemic alterations on the root metabolomic signature triggered by *M. incognita*. We performed an untargeted metabolomic analysis of roots upon shoot and root herbivory. Principal component analysis (PCA) was used as an unsupervised method to produce interpretable projections of the samples in a reduced dimensionality (scores plot). At 3 days after nematode inoculation, the first two principal components (PCs) explained 55.3 % of the total variance (Fig. 7a). Roots inoculated with *M. incognita* were separated from control roots along with the PC1, which explained about 35.3 % of the variation. *M. incognita* roots were also separated from *M. sexta* roots and *M. incognita* plus *M. sexta* roots on the PC1 and PC2 (the last one explaining 20 % of the total variation). *M. sexta* roots and *M. incognita* plus *M. sexta* roots separated from control roots in the PC1 and PC2. However, there was an overlap between roots of plants inoculated with *M. sexta* plus *M. incognita* and roots of plants inoculated with *M. sexta* alone, and

no separation was found between these samples (Fig. 7a). At 7 days after *M. incognita* inoculation, the top two PCs explained about 50 % of the total variance (Fig. 7b). There was no clear separation between the roots of plants inoculated with *M. incognita* and control plants. However, PC2, which explained 13.6 % of the variation, separated *M. incognita* roots from *M. sexta* roots and partially from *M. incognita* plus *M. sexta* roots (Fig. 7b). PC2 also separated control roots from *M. sexta* roots and partially from roots of plants inoculated with *M. incognita* plus *M. sexta*. However, there was not a clear separation between *M. sexta* roots and *M. incognita* plus *M. sexta* roots (Fig. 7b). At 21 days after nematode inoculation, there was no clear separation in the PCA between the different samples (Fig. 7c). Our data indicate that at early time points (namely 3 and 7 days), *M. sexta* leaf herbivory impacts systemically the root metabolomic signature triggered by *M. incognita* infection.

**Leaf herbivory by *Manduca sexta* counteracts the ability of *Meloidogyne incognita* to decrease root levels of steroidal glycoalkaloids, a polyamine conjugate, and a chlorogenic acid dimer**

To predict metabolite structures that could explain the separation in the PCA between the treatments (Fig. 7), we interpreted the mass spectra of the most variable loadings and hypothesized structures when possible, as described above. We selected the time points 3 and 7 days after *M. incognita* infection as they showed a stronger separation between the treatments (Fig. 7). We were able to hypothesize the following structures: *α*-tomatine; *α*-dehydrotomatine; hydroxylated δ-tomatine; a polyamine conjugated to a phenylpropanoid and a chlorogenic acid dimer (Fig. 8; Fig. S3).

After predicting the structures of the metabolites, we studied whether *M. incognita* infection affected the levels of these predicted metabolites in tomato roots. We compared the intensity of a diagnostic fragment for each predicted molecule. *M. incognita* decreased the levels of the steroidal glycoalkaloid α-tomatine in tomato roots at 3 and 7 days after the infection (Fig. 8a, Table S4, S5). At 21 days after the infection, α-tomatine levels in *M. incognita* infected roots were similar to that in control roots (Fig. 8a). Similarly, *M. incognita* also decreased the levels of the steroidal glycoalkaloid *α*-dehydrotomatine at 3 days after inoculation (Fig. 8b). However, no differences in *α*-dehydrotomatine levels were found between *M. incognita* infected and control roots at 7 and 21 days (Fig. 8b). *M. incognita* also decreased the levels of the steroidal glycoalkaloid hydroxylated δ-tomatine 3 days after inoculation (Fig. 8c). However, there were no differences in the levels of hydroxylated δ-tomatine at 7 days between *M. incognita* infected roots and control roots. At 21 days after inoculation, *M. incognita* increased the levels of hydroxylated δ-tomatine in tomato roots (Fig. 8c). *M. incognita* infection strongly decreased the levels of a polyamine conjugated to a phenylpropanoid at 3 and 7 days (Fig. 8d). However, at 21 days after inoculation, we did not find differences in the levels of this polyamine conjugate between *M. incognita* infected roots and control roots (Fig. 8d).

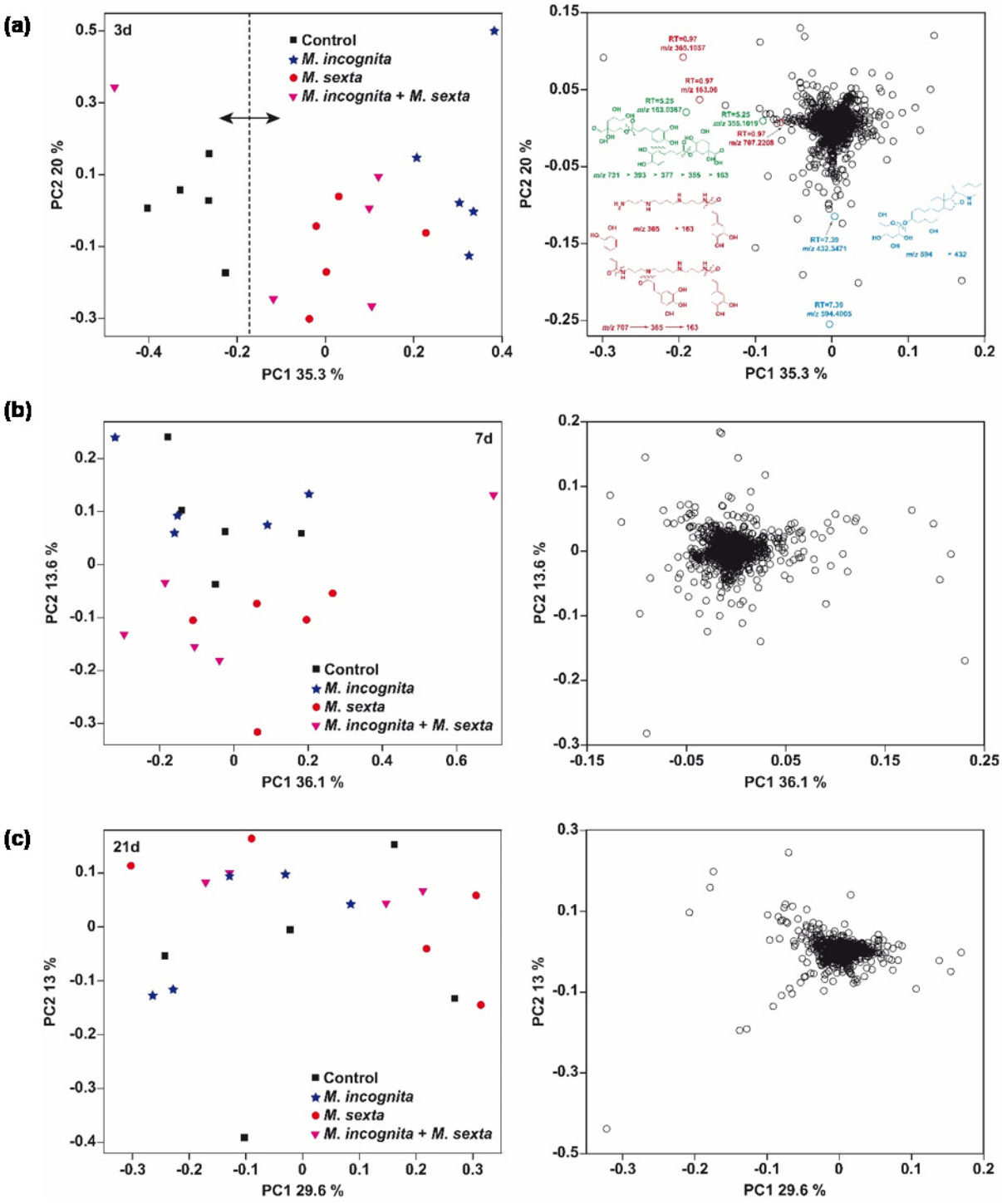


**Figure 7**. **Impact of *Meloidogyne incognita* and *Manduca sexta* on the metabolomics profile of tomato roots.** Principal component analysis (PCA) of the global metabolome of tomato roots (scores, left panels) and loading plots displaying the projection of each LC-MS feature (right panels). Plants were challenged with *M. incognita* or *M. sexta* alone or in combination, or not challenged (control). Metabolite profiles were analysed (a) 3 days, (b) 7 days and (c) 21 days after *M. incognita* inoculation. Arrows in panel (a) point to the most variable loadings selected for structural prediction.

*M. incognita* also strongly decreased the levels of the chlorogenic acid dimer at 3 and 7 days (Fig. 8e). Moreover, although to a lesser extent, at 21 days, the levels of the chlorogenic acid dimer in *M. incognita* infected plants remained lower compared to control plants (Fig. 8e). These results show the ability of *M. incognita* to decrease the levels of the predicted metabolites, mostly during early stages of the infection.

We next studied whether *M. sexta* leaf herbivory systemically affects *M. incognita* ability to decrease the defense-related metabolites in tomato roots. *M. sexta* herbivory did not significantly affect the root levels of α-tomatine (Fig. 8a). Similarly, *M. sexta* did not alter the levels of *α*-dehydrotomatine at 3 and 21 days after the challenge. However, an increase in *α*-dehydrotomatine levels was found in *M. sexta* challenged plants, 7 days after herbivory (Fig. 8b). *M. sexta* herbivory did not significantly affect the root levels of hydroxylated δ-tomatine at 3 days after herbivory. However, an increase in the level of hydroxylated δ-tomatine was found in *M. sexta*-challenged plants at 7 and 21 days after herbivory (Fig. 8c). Although to a lesser extent compared to *M. incognita* root infection, *M. sexta* leaf herbivory also led to a decrease in the levels of the polyamine conjugate and the chlorogenic acid dimer, at 3 days after herbivory (Fig 8d,e). However, at 7 and 21 days, no differences in the level of the polyamine conjugate and the chlorogenic acid dimer were found between *M. sexta* and control roots (Fig. 8d,e). Remarkably, when plants were inoculated with both *M. sexta* and *M. incognita*, *M. incognita* failed partially or entirely in reducing the levels of the analyzed metabolites (Fig. 8). Moreover, the level of the analyzed metabolites in the root of co-infected plants remained in general, more similar to those observed in roots plant challenged with *M. sexta* alone than to those found in plants challenged with *M. incognita* alone (Fig. 8).

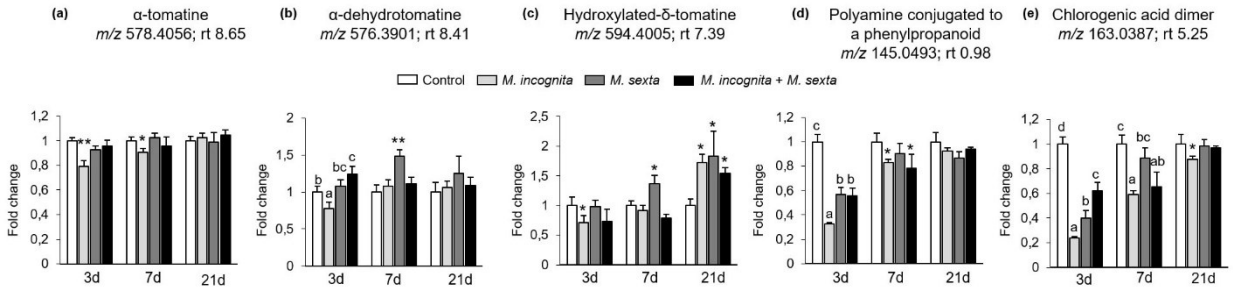


**Figure 8**. ***Manduca sexta* leaf herbivory antagonizes the repression on the accumulation of defense-related metabolites triggered by *Meloidogyne incognita*.** Fold changes of the selected metabolites (a) *α*-tomatine; (b) *α*-dehydrotomatine; (c) Hydroxilated δ-tomatine; (d) polyamine conjugate; and (e) chlorogenic acid dimer in roots of tomato plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Metabolites were analysed at 3, 7 and 21 days after *M. incognita* inoculation. Data are mean + SE. Intensity data are expressed relative to those found in control plants at each sampling time, which were arbitrarily given a value of 1. At each time point different letters indicate differences between treatments according to Tukey's test following two-way ANOVA with factors *M. incognita* challenge and *M. sexta* challenge (Table S4). At each time point asterisks mean significantly different from control plants according to Dunnett's test ($P < 0.05$, n=5). *, $P < 0.05$; ** $P < 0.01$.

## DISCUSSION

We demonstrated that continuous leaf herbivory by *M. sexta* reduces the performance of the RKN *M. incognita via* shoot-to-root interaction. By using a series of manipulative bioassays in which we incorporated the shoot herbivore at different stages of the nematode infection cycle, we found that *M. sexta* leaf herbivory delayed *M. incognita* root invasion, and impaired the development (galling) and fecundity inside plant roots. Several studies have demonstrated that leaf herbivory or shoot elicitation with jasmonates can affect the susceptibility of roots to RKN, facilitating or impeding nematode performance depending on the study systems and the specific parameters addressed. For instance, tobacco shoot defoliation by *M. sexta* increased the number of *M. incognita* eggs per gram of root (Kaplan *et al*., 2008). Along the same lines, shoot elicitation with simulated herbivory increased the number of *M. incognita* eggs, while it did not affect the number of galls in tobacco plants (Machado *et al*., 2018). By contrast, a reduction in the number of *M. incognita* galls was found in roots of plants that were previously challenged with aphids (Kafle *et al*., 2017) or shoot elicited with methyl jasmonate (Nahar *et al*., 2011; Vieira dos Santos *et al*., 2013). The plant interaction with RKN is highly complex and dynamic (Goverse & Bird, 2011; Goverse & Smant, 2014; Ibrahim *et al*., 2019). Therefore, such variability of results could be attributed to the differences in the study systems, experimental designs and/or sampling times (Wondafrash *et al*., 2013; van Dam *et al*., 2018; Ibrahim *et al*., 2019). Here, by performing a systematic study, we demonstrated that *M. sexta* continuous shoot herbivory negatively affected *M. incognita* root infection throughout the entire nematode infection cycle.

Jasmonates are important regulatory signals in plant-mediated interactions between leaf- and root-feeding herbivores (Erb *et al*., 2009; van Dam *et al*., 2011; Machado *et al*., 2013; Fragoso *et al*., 2014; Li *et al*., 2017; Wang *et al*., 2019). To assess the involvement of jasmonates in *M. sexta-M. incognita* shoot-to-root interactions, we used grafted plants compromised in jasmonate biosynthesis or perception. By restraining jasmonate impairment to the shoot, we were able to identify the specific contribution of the aboveground jasmonate pathway in *M. sexta-M. incognita* shoot-to-root interactions. The grafting experiments showed that the negative impact of *M. sexta* herbivory on *M. incognita* infection does not require *de novo* jasmonates biosynthesis in tomato shoots. Leaf herbivory still reduced the number of galls in wt roots grafted with shoots that were compromised in wound-induced jasmonate biosynthesis (*spr2* and *def1*). It is noteworthy that Machado *et al*. (2018) recently found that the jasmonate biosynthesis pathway was required in intact tobacco plants for shoot-to-root herbivorous interaction. The authors found that the increased *M. incognita* egg number triggered by simulated shoot herbivory was abolished in *irAOC* plants, compromised in jasmonate biosynthesis. However, in their study the authors did not restrain jasmonate biosynthesis impairment to the shoot organs. Therefore it was not possible to discern whether jasmonates biosynthesis was required in shoots and/or roots for the shoot-to-root interaction. By contrast to the

jasmonate biosynthesis pathway, we found that leaf herbivory did not reduce the number of root galls in grafts with shoots compromised in jasmonate perception (*jai1*). These findings indicate the requirement of intact jasmonate perception for leaf herbivory-triggered root impairment of *M. incognita* infection. It is noteworthy that leaf herbivory led to an increase in the number of root galls in grafts with shoots compromised in jasmonate perception. Although we do not have a specific explanation for this phenotype, it suggests that other mechanisms, independent of jasmonate signaling, are involved in this shoot-to-root interaction. The impact of leaf herbivores on root parasitic nematodes likely depends on the balance between positive effects resulting from increased carbohydrate allocation to the roots (Kaplan *et al*., 2009; Biere & Goverse 2016) and negative effects resulting from the elicitation of root defenses (Bhattarai *et al*., 2008; Nahar *et al*., 2011; Kyndt *et al*. 2017). Together, our results suggest that an intact jasmonate perception pathway but not jasmonate biosynthesis pathway in shoots is required for the negative systemic effect of *M. sexta* herbivory on *M. incognita* performance.

A growing body of evidence demonstrates a pivotal role of jasmonate-regulated defenses in the immune responses against RKNs (Cooper *et al*., 2005; Fujimoto *et al*., 2011; Nahar *et al*., 2011; Vieira dos Santos *et al*., 2013; Fan *et al*., 2015; Zhao *et al*., 2015; Zhou *et al*., 2015; Gleason *et al*., 2016; Kyndt *et al*., 2017). Accordingly, we found that *M. incognita* infection led to the repression of the13-LOX branch of the oxylipin pathway, which leads to the family of jasmonates. Remarkably, this repression was stronger during the early stages of the nematode infection cycle (invasion and induction stages). Previous studies evidenced the ability of RKNs to repress jasmonate-related root defenses at the very early stages after penetration, probably to promote infection success (Barcala *et al*., 2010; Kyndt *et al*., 2012; Nahar *et al*., 2011; Ji *et al*., 2013; Iberkleid *et al*., 2015; Gheysen & Mitchum, 2019). Indeed, a stronger repression of *LOXD* was found in tomato roots during the early stages of *Meloidogyne javanica* infection, compared to later stages of the infection (Iberkleid *et al*., 2015). In contrast to *M. incognita*, *M. sexta* leaf herbivory triggered a strong activation of the jasmonate biosynthesis pathway in roots. It was previously demonstrated that leaf herbivory or mechanical wounding triggers jasmonate-related responses systemically in root tissues (Acosta *et al*., 2013; Machado *et al*., 2013; Larrieu *et al*., 2015; Machado *et al*., 2018; Schulze *et al*., 2019). Interestingly, in roots of plants that were co-infected, leaf herbivory prevented the root repression of the jasmonate biosynthesis pathway triggered by *M. incognita* infection. It was recently found that root inoculation with the beneficial fungi *Trichoderma harzianum* antagonized the ability of *M. incognita* to downregulate jasmonate-related responses, by priming jasmonate-related defenses in systemic roots (Martinez-Medina *et al*., 2016, 2017). In the same line Nahar *et al*. (2011) found that shoot elicitation with methyl jasmonate antagonized the *Meloidogyne gaminicola*-induced defense gene repression in roots of rice plants. It is thus tempting to speculate that the boost of jasmonate-related responses triggered in roots

by leaf herbivory might interfere with the nematode's ability for manipulating jasmonate-related defenses, leading to higher plant resistance to nematodes.

Besides jasmonates, the 9-LOX branch of the oxylipin pathway has been associated with plant resistance to RKNs (Gao *et al*., 2007; Iberkleid *et al*., 2015). We found that *M. incognita* infection did not significantly affect the expression of the gene markers for the 9-LOX pathway in roots. By contrast, leaf herbivory triggered a general activation of the 9-LOX branch of the oxylipin pathway in roots. Similarly, the root of plants that were co-infected with the root and leaf herbivores showed increased activation of the 9-LOX pathway. Several studies support a role of oxylipins produced by the 9-LOX pathway in root defenses. For instance, the 9-LOX derivative 9-hydroxyoctadecatrienoic acid (9-HOT) is involved in cell wall modification and ROS signaling in roots (Vellosillo *et al*., 2007; Marcos *et al*., 2015). It is therefore conceivable that the activation of the 9-LOX pathway in roots by leaf herbivory could participate in the increased resistance to *M. incognita*. However, the specific role of the 9-LOX branch of the oxylipins pathway in root-nematode interactions remains unknown so far.

Jasmonates regulate nearly all biosynthetic pathways leading to secondary metabolites (Wasternack & Strnad, 2019). According to the significant impact of *M. incognita* on root jasmonates, *M. incognita* infection triggered significant changes in root secondary metabolism. Similarly, previous studies reveal the strong impact of parasitic nematodes on the global metabolome of their host plants, including changes in defensive compounds and primary metabolism (Hofmann *et al*., 2010; Eloh *et al*., 2016; Machado *et al*., 2018; Willett *et al*., 2020). Following the dynamic observed on the nematode effect on root jasmonates, a stronger impact on the root metabolome was found during the early stages of infection compared to later stages. Indeed, *M. incognita* infection at 3 days after inoculation reduced the root levels of the steroidal glycoalkaloids *α*-tomatine, *α*-dehydrotomatine, and hydroxylated δ-tomatine. Steroidal glycoalkaloids are jasmonate-regulated defensive compounds with antiherbivore properties (Altesor *et al*., 2014; Chowanski *et al*., 2016; Abdelkareem *et al*., 2017; Montero-Vargas *et al*., 2018; Calf *et al*., 2018). Though the involvement of steroidal glycoalkaloids on plant-nematode interactions remains ambiguous, several reports reveal the nematicidal activity of different alkaloids in different plant species (Thoden *et al*., 2009; Wang *et al*., 2012; Jang *et al*., 2015). These studies suggest that the accumulation of glycoalkaloids may have an important role in root immunity against nematode attack. Besides steroidal glycoalkaloids, *M. incognita* infection also decreased the levels of a polyamine conjugate and a chlorogenic acid dimer. In this case, the reduction was also found at later stages of the nematode infection cycle. Polyamines and polyphenols as chlorogenic acid are prominent defense metabolites against a broad range of insect herbivores (Bassard *et al*., 2010; Kaur *et al*., 2010; Macoy *et al*., 2015; Kundu & Vadassery, 2019). Several reports further indicate that polyamines and polyphenols are involved in plant resistance against parasitic nematodes (Pegard *et al*., 2005; Heinick *et al*., 2010; Hewezi *et al*., 2010). Accordingly, the root repression in the accumulation of

the steroidal glycoalkaloids, the polyamine conjugate, and the chlorogenic acid dimer triggered by *M. incognita* attack would favor the nematode infection success.

Interestingly, we found that *M. sexta* leaf herbivory systemically altered the metabolomic signature triggered in roots by *M. incognita* infection. The impact of shoot herbivory on root global metabolome has been previously demonstrated (Marti *et al*., 2013; Gulati *et al*., 2014; Machado *et al*., 2018). Our results further demonstrate the strong influence of aboveground elicitation in the root responses deployed against root herbivores. Indeed, *M. sexta* leaf herbivory prevented, totally or partially, the repression in the accumulation of the defense-related metabolites triggered by early *M. incognita* infection. These findings indicate that *M. sexta* leaf herbivory interferes systemically with the ability of *M. incognita* for repressing the accumulation of defensive compounds in roots, contributing to a stronger anti-nematode defense response.

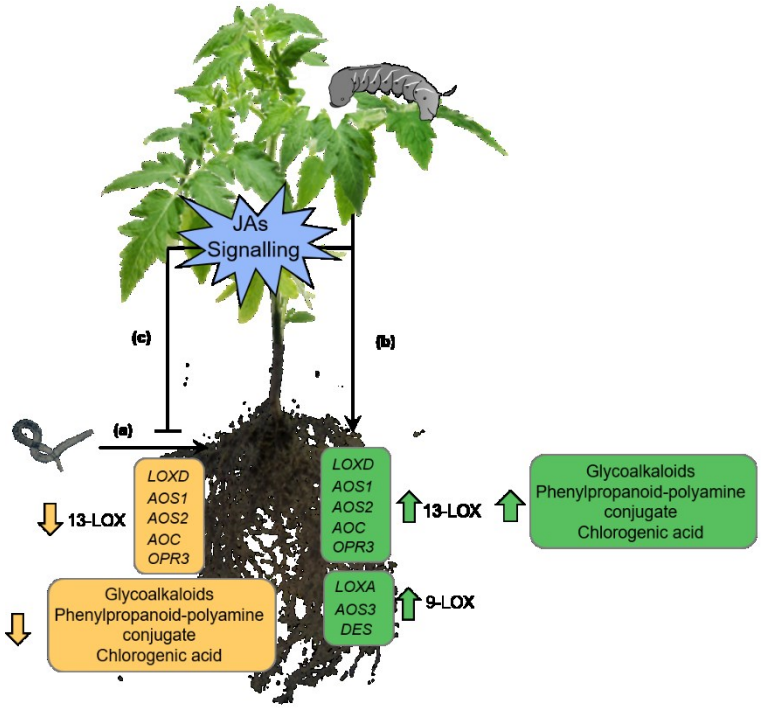


**Figure 9. Model for the shoot-to-root impact of *Manduca sexta* herbivory on *Meloidogyne incognita* infection.** (a) Root infection by *M. incognita* leads to an early and transient downregulation of the 13-LOX branch of oxylipin pathway and the repression of defense-related metabolites in tomato roots. The yellow boxes show specific genes marker for the 13-LOX pathway and defense metabolites that are repressed by *M. incognita* infection. (b) *M. sexta* leaf herbivory triggers a systemic activation of the 13-LOX and 9-LOX branches of the oxylipin pathway, and the accumulation of defense-related metabolites in tomato roots. The green boxes show specific genes marker for the 13-LOX and 9-LOX branches of the oxylipin pathway, and defense metabolites that are enhanced in roots by *M. sexta* leaf herbivory. (c) *M. sexta* leaf herbivory further antagonizes the *M. incognita*-triggered repression of the13-LOX branch of oxylipin pathway and defense-related metabolites in tomato roots, leading to a higher nematode resistance. The shoot jasmonate signaling pathway mediate the impact of *M. sexta* leaf herbivory on *M. incognita* root infection.

In conclusion, our study shows that leaf herbivory profoundly alters the defense-related responses triggered in roots by RKNs (Fig. 9). Our findings further indicate that *M. sexta* leaf herbivory interferes, directly or indirectly, with *M. incognita*'s ability to suppress root defenses. This leads to a delayed nematode invasion and reduced gall formation and fecundity. In addition, our results highlight the importance of the shoot jasmonate perception pathway and the independence of *the novo* shoot jasmonates biosynthesis in the *M. sexta-M. incognita* shoot-to-root interaction.

## ACKNOWLEDGMENTS

We acknowledge the support of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig funded by the German Research Foundation (FZT 118). CMM thanks the Deutscher Akademischer Austauschdienst (DAAD) for a doctoral research grant (research grant No: 91607343). AMM further acknowledges support from the program for attracting talent to Salamanca from Fundación Salamanca Ciudad de Cultura y Saberes and Ayuntamiento de Salamanca. We acknowledge Daniel Uhlig for support in the glasshouse bioassays.

## AUTHOR CONTRIBUTION

**AMM** and **NMvD** planned and designed the research. **AMM** and **CM** performed experiments. **AMM, CMM, AM, AW** and **FV** analysed data. **AMM** and **NMvD** wrote the manuscript with input from all the authors.

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**SUPPORTING INFORMATION**

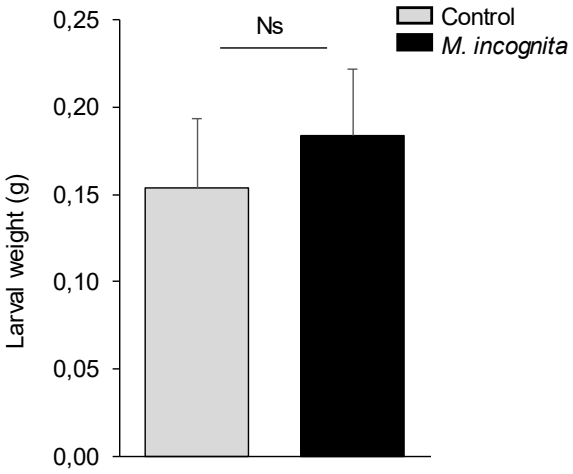


**Figure S1: Impact of *Meloidogyne incognita* root infection on *Manduca sexta* larval weight.** *M. sexta* larval weigh was measured in larvae feeding for seven days on control plants or on plants root infected with *M. incognita*. Data are means ± SE (n =15 biological replicates). NS: not significant.

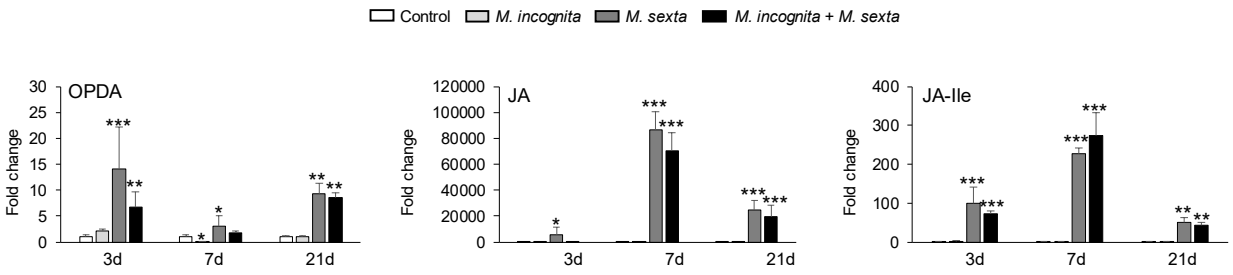


**Figure S2: Impact of *Meloidogyne incognita* and *Manduca sexta* on shoot jasmonates content.** Shoot levels of OPDA (*oxo*phytodienoic acid) JA (jasmonic acid) and JA-Ile (jasmonyl-*L*-isoleucine) were analyzed in shoots of plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Jasmonates were analyzed at 3, 7, and 21 days after *M. incognita* inoculation. Data are mean + SE. The accumulation levels are expressed relative to those found in control plants at each sampling time, which were arbitrarily given a value of 1. At each time point, asterisks mean significantly different from control plants, according to Dunnett's test ($P < 0.05$, n=5). *, $P < 0.05$; **, $P < 0.01$.

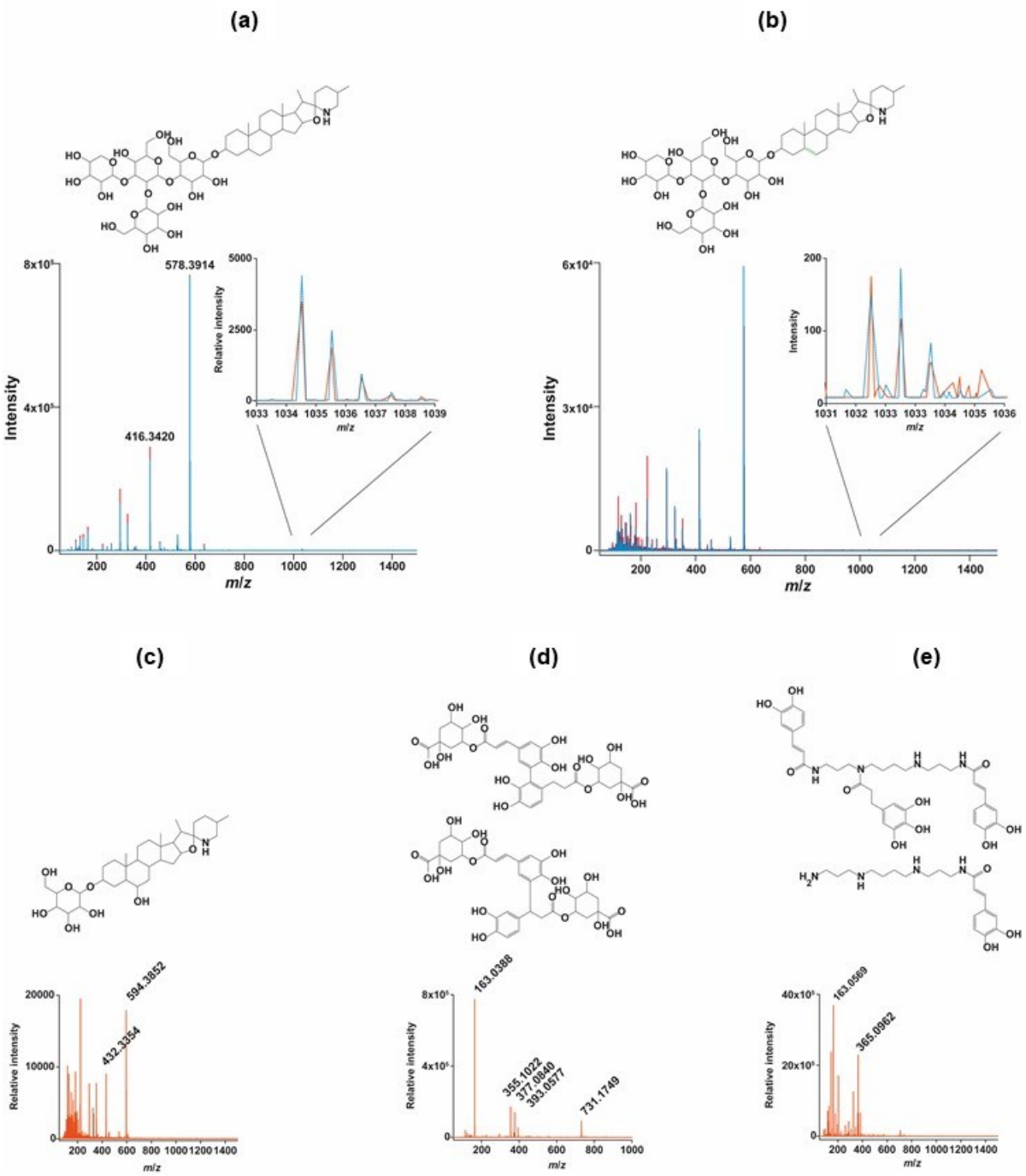


**Figure S3: Mass spectra and structures of the predicted metabolites.** (a) *α*-tomatine; (b) *α*-dehydrotomatine; (c) Hydroxylated δ-tomatine; (d) Chlorogenic acid dimer; (e) Polyamine conjugated to a phenylpropanoid

**Table S1** Primer sequences used for the real-time qPCR analysis

| ID | Target Gene | Primer (5´-3´) |
|---|---|---|
| U37840 | *Lypoxygenase D* (*LOXD*)[1] | GACTGGTCCAAGTTCACGATCC<br>ATGTGCTGCCAATATAAATGGTTCC |
| AJ271093 | *Allene oxide synthase* (*AOS1*)[1] | CACCTGTTAAACAAGCGAAAC<br>GACCTGGTGGCATGTTCGT |
| AF230371 | *Allene oxide synthase 2* (*AOS2*)[1] | AGATTTTCTTCCCGAATATGCTGAA<br>ATACTACTGATTTCATCAACGGCAT |
| AF384374 | *Allene oxide cyclase* (*AOC*)[1] | GCACGAAGAAGAGAAGAAAGGAGAT<br>CGGTGACGGCTAGGTAAGTTTC |
| AJ278332 | *12-oxophytodienoic acid reductase 3* (*OPR3*)[1] | TTGGCTTAGCAGTTGTTGAAAG<br>TACGTATCGTGGCTGTGTTACA |
| U09026 | *Lypoxygenase A* (*LOXA*)[1] | GGTTACCTCCCAAATCGTCC<br>TGTTTGTAACTGCGCTGTG |
| AF454634 | *Allene oxide synthase 3* (*AOS3*)[1] | GCGGAGGAGTTCAATCCAG<br>CGCATGAAAAACTCCACAACC |
| AF317515 | *Divinyl Ether Synthase* (*DES*)[1] | CCGGATGAGTTTGTACCTGA<br>ATCTTTGCCTGGACATTGCT |
| X14449 | Elongation factor 1α (SlEF)[1] | GATTGGTGGTATTGGAACTGTC<br>AGCTTCGTGGTGCATCTC |
| MINC06773a | *M. incognita* Actin[2] | GATGGCTACAGCTGCTTCGT<br>GGACAGTGTTGGCGTAAAGG |

[1]López-Ráez et al., 2010; [2]Teillet *et al*., 2013.

**Table S2**: Results of two-way ANOVAs for the response of the root expression of *LOXD*, *AOS1*, *AOS2*, *AOC2*, *OPR3*, *OPDA*, *LOXA*, *AOS3*, *DES1* and root content of OPDA, JA and JA-Ile to *Manduca sexta* and *Meloidogyne incognita* infection. Statistically significant effects are indicated in bold.

| | *Manduca sexta* (M.s.) | *Meloidogyne incognita* (M.i.) | M.s.*M.i. |
|---|---|---|---|
| Variable | *P value* | *P value* | *P valu* |
| *LoxD* 3d | **0.007** | **0.048** | **0.026** |
| *LoxD* 7d | **0.002** | **0.05** | 0.171 |
| *LoxD* 21d | **0.031** | 0.145 | 0.115 |
| *AOS1* 3d | **0.008** | 0.908 | 0.783 |
| *AOS1* 7d | **0.019** | **0.031** | **0.049** |
| *AOS1* 21d | **<0.001** | 0.131 | **0.048** |
| *AOS2* 3d | 0.501 | **0.037** | 0.439 |
| *AOS2* 7d | **<0.001** | **0.009** | **0.048** |
| *AOS2* 21d | **0.089** | 0.202 | 0.472 |
| *AOC* 3d | **<0.001** | 0.853 | 0.351 |
| *AOC* 7d | **<0.001** | **<0.001** | **0.041** |
| *AOC* 21d | **0.002** | 0.085 | 0.176 |
| *OPR3* 3d | **<0.001** | **0.05** | 0.304 |
| *OPR3* 7d | **<0.001** | **<0.001** | **0.017** |
| *OPR3* 21d | **0.018** | 0.122 | **0.042** |
| OPDA 3d | **<0.001** | **0.047** | 0.246 |
| OPDA 7d | **0.03** | 0.317 | 0.246 |
| OPDA 21d | **0.032** | 0.139 | **0.038** |
| JA 3d | **0.004** | **0.033** | **0.0472** |
| JA 7d | 0.150 | **0.021** | **0.019** |
| JA 21d | **0.018** | 0.074 | 0.355 |
| JA-Ile 3d | 0.462 | 0.057 | 0.4531 |
| JA-Ile 7d | 0.131 | 0.324 | 0.594 |
| JA-Ile 21d | **0.05** | 0.135 | **0.002** |
| *LOXA* 3d | **0.039** | 0.206 | 0.330 |
| *LOXA* 7d | **<0.001** | 0.125 | 0.250 |
| *LOXA* 21d | **0.019** | **0.046** | 0.321 |
| *AOS3* 3d | **0.045** | 0.251 | 0.254 |
| *AOS3* 7d | **0.002** | 0.116 | 0.976 |
| *AOS3* 21d | **0.010** | 0.056 | 0.465 |
| *DES1* 3d | **<0.001** | **0.028** | **0.001** |
| *DES1* 7d | **<0.001** | **0.0150** | **0.028** |
| *DES1* 21d | **<0.001** | 0.681 | 0.163 |

**Table S3**: Root levels of OPDA, JA and JA-Ile (ng/mg Fw) in roots of plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Hormone contents were analyzed at 3, 7 and 21 days after *M. incognita* inoculation.

| Treatment | OPDA | JA | JA-Ile |
|---|---|---|---|
| Control 3d | 1,20 ± 0,24 | 16,76 ± 0,98 | 1,04 ± 0,32 |
| *M. incognita* 3d | 0,75 ± 0,25 | 11,73 ± 0,54 | 2,94 ± 0,89 |
| *M. sexta* 3d | 2,15 ± 0,33 | 22,28 ± 2,09 | 2,03 ± 0,38 |
| *M. incognita* + *M. sexta* 3d | 2,32 ± 0,44 | 18,66 ± 2,86 | 2,93 ± 0,81 |
| Control 7d | 1,02 ± 0,13 | 4,82 ± 0,72 | 0,37 ± 0,11 |
| *M. incognita* 7d | 1,05 ± 0,20 | 2,54 ± 0,33 | 0,27 ± 0,08 |
| *M. sexta* 7d | 0,84 ± 0,17 | 4,77 ± 0,73 | 0,83 ± 0,40 |
| *M. incognita* + *M. sexta* 7d | 0,49 ± 0,11 | 5,40 ± 0,52 | 0,49 ± 0,12 |
| Control 21d | 0,87 ± 0,31 | 70,97 ± 12,87 | 29,46 ± 5,22 |
| *M. incognita* 21d | 0,65 ± 0,13 | 113,36 ± 7,86 | 42,90 ± 5,38 |
| *M. sexta* 21d | 0,89 ± 0,21 | 131,51 ± 33,71 | 65,81 ± 14,40 |
| *M. incognita* + *M. sexta* 21d | 2,04 ± 0,49 | 155,08 ± 29,39 | 32,80 ± 1,76 |

**Table S4**: Relative intensity of the selected metabolites in roots of plants that were challenged with *M. incognita* or *M. sexta* alone or in combination, and in not challenged control plants. Relative intensity was analyzed at 3, 7 and 21 days after *M. incognita* inoculation. For each predicted structure, the mass to charge ratio (*m*/*z*) and the retention time (rt) in minutes is indicated.

| Treatment | α-tomatine *m/z* 578.4056; rt 8.65 | α-dehydrotomatine *m/z* 576,3901; rt 8,41 | Hydroxylated δ-tomatine *m/z* 594,4005; rt 7,39 | Polyamine conjugate *m/z* 145,0493; rt 0,98 | Chlorogenic acid *m/z* 163,0387; rt 5,25 |
|---|---|---|---|---|---|
| Control 3d | 14505427,2 ± 368557,063 | 1683473,8 ± 127534,813 | 1043639,2 ± 150017,981 | 489286 ± 29266,4496 | 595839,2 ± 42196,6345 |
| *M. incognita* 3d | 11513734,6 ± 675978,114 | 1309136,4 ± 145135,333 | 744902,2 ± 125164,61 | 158824,8 ± 5747,39035 | 141557 ± 22076,4696 |
| *M. sexta* 3d | 13471726,2 ± 401648,024 | 1817308,6 ± 139903,73 | 1022533,4 ± 118296,452 | 276840,4 ± 29119,8897 | 238508,4 ± 39728,9032 |
| *M. incognita* + *M. sexta* 3d | 13922645,4 ± 645472,447 | 2100155,6 ± 173223,215 | 761564,8 ± 164507,362 | 273352,2 ± 31023,7655 | 372226,6 ± 100976,126 |
| Control 7d | 14209563,3 ± 361081,52 | 1501966,25 ± 121750,56 | 692847,5 ± 46289,4076 | 187897,6 ± 13845,8928 | 328517,4 ± 36615,592 |
| *M. incognita* 7d | 12887725,8 ± 391499,468 | 1619667 ± 109734,053 | 635289,75 ± 49442,2578 | 155661,75 ± 4611,82154 | 194674 ± 17457,3731 |
| *M. sexta* 7d | 14564652,4 ± 535884,369 | 2236166,5 ± 111694,972 | 946850,75 ± 87009,435 | 169592 ± 15717,9569 | 291135 ± 19932,4071 |
| *M. incognita* + *M. sexta* 7d | 13616402,4 ± 995083,452 | 1666568,25 ± 119758,162 | 543892,25 ± 36402,9255 | 146830,2 ± 21860,4217 | 215581,6 ± 51292,8236 |
| Control 21d | 3092355,25 ± 126166,573 | 1427819,33 ± 115394,319 | 418806 ± 27808,9686 | 1677806,2 ± 79573,3024 | 363365,25 ± 27571,0024 |
| *M. incognita* 21 d | 3250073,6 ± 152461,886 | 1510484 ± 109334,846 | 723329 ± 55687,5482 | 1529836,4 ± 38925,4859 | 317955,5 ± 6918,02196 |
| *M. sexta* 21d | 3358873,2 ± 209227,913 | 1555296,75 ± 141788,136 | 767907 ± 175745,861 | 1476632,8 ± 87216,2772 | 357489,4 ± 39175,6984 |
| *M. incognita* + *M. sexta* 21d | 3149548,2 ± 350238,158 | 1786104,6 ± 340315,635 | 643199,5 ± 68923,1661 | 1585276,4 ± 56835,2222 | 352918 ± 12202,2665 |

**Table S5**: Results of two-way ANOVAs for the response of the root content of *α*-tomatine, *α*-dehydrotomatine, hydroxylated δ-tomatine, polyamine conjugated to a phenylpropanoid and chlorogenic acid dimer to *Manduca sexta* and *Meloidogyne incognita* infection. Statistically significant effects are indicated in bold.

| | *Manduca sexta* (M.s.) | *Meloidogyne incognita* (M.i.) | M.s.*M.i. |
|---|---|---|---|
| Variable | *P value* | *P value* | *P value* |
| -tomatine 3d | 0.222 | **0.032** | **0.006** |
| *α*-tomatine 7d | 0.398 | **0.05** | 0.769 |
| *α*-tomatine 21d | 0.719 | 0.911 | 0.430 |
| *α*-dehydrotomatine 3d | 0.760 | **0.006** | **0.041** |
| *α*-dehydrotomatine 7d | **0.004** | 0.069 | **0.009** |
| *α*-dehydrotomatine 21d | 0.330 | 0.446 | 0.136 |
| Hydroxylated δ-tomatine 3d | 0.988 | **0.049** | 0.895 |
| Hydroxylated δ-tomatine 7d | **0.001** | 0.180 | **0.009** |
| Hydroxylated δ-tomatine 21d | **0.049** | **0.046** | 0.379 |
| Polyamine conjugate 3d | 0.05 | **<0.001** | **<0.001** |
| Polyamine conjugate 7d | 0.389 | **0.048** | **0.049** |
| Polyamine conjugate 21 d | 0.302 | 0.079 | 0.777 |
| Chlorogenic acid dimer 3d | **0.030** | **0.01** | **<0.001** |
| Chlorogenic acid dimer 7d | **0.049** | **0.007** | **0.042** |
| Chlorogenic acid dimer 21d | 0.568 | **0.05** | 0.331 |

**Material and Methods S1: Metabolites extraction and analysis**

We extracted 100 mg fresh ground root tissue of each sample in 1 ml of extraction buffer (75 % methanol acetate buffer; pH 4.8; diluted 1:5, v: v). The samples were homogenized for 5 min at 30 Hz using a ball mill (Retsch mixer mill MM 400), and subsequently centrifuged (25,155 g, 10 min, 4 °C). The supernatant was collected in a 2 ml Eppendorf tube. We repeated the extraction procedure with the remaining pellet and combined the supernatant with the first one. We centrifuged (25,155g, 5 min, 4°C) all extracts, transferred 200 µl of each to an HPLC vial and added 800 µl extraction buffer, resulting in a 1 : 5 dilution. We performed chromatographic separation of all diluted extracts by injecting 2 µl on a Thermo Scientific Dionex UltiMate 3000 UPLC (Thermo Scientific Dionex, Sunnyvale, USA), equipped with a C18 column (Acclaim RSLC 120 C18, 2.2 µm, 120 Å, 2.1 x 150 mm, Thermo Fisher Scientific). We applied the following binary elution gradient at a column temperature of 40 °C; and a flow rate of 0.4 ml min-1 : 0–2 min, 95 % A (water and 0.05 % formic acid), 5 % B (acetonitrile and 0.05 % formic acid); 2–12 min, 5 to 50 % B; 12–13 min, 50 to 95 % B; 13–15 min, 95 % B; 15–16 min, 95 to 5 % B; 16–20 min, 5 % B. Metabolites were analyzed on a quadrupole/time-of-flight mass spectrometer (qToF-MS; Bruker maXis impact HD; Bruker Daltonik, Bremen, Germany) with an electrospray ionization source operated in positive mode. Instrument settings were as follows: capillary voltage, 2,500 V; nebulizer, 2.5 bar; dry gas temperature, 220 °C; dry gas flow, 11 L $min^{-1}$; scan range, 50–1000 *m*/*z*; acquisition rate, 1 Hz. We used sodium formate clusters (10 mM solution of NaOH in 50/50 % [v/v] isopropanol/water containing 0.2 % formic acid) to perform mass calibration. A commercial standard of α-tomatine (Sigma) was injected with the same conditions described above but the scan range was modified to 50–1500 *m*/*z*.

**Material and Methods S2: Data processing of the liquid chromatography mass spectrometry**

We transformed the LC-qToF-MS raw data *.mzXML* files with the programs CompassXport and DataAnalysis v4.2 SR2 (Bruker Daltonik GmbH). We converted the *.mzXML* files into *.abf* files with the software Reifycs Abf Converter (RIKEN, https://www.reifycs.com/AbfConverter/). We deconvoluted the LC-qToF-MS data stored in the *.abf* files with the software MS-DIAL v3.08 from RIKEN (Lai 2018). Processing parameters were as follows: soft ionization, centroid data type, positive ion mode, (*i*) data collection: mass accuracy MS1 = 0.01 Da, retention time 0.7–14 min, mass range 45-1005 Da; (*ii*) peak detection: minimum peak height (amplitude) = 1,000, mass slice width = 0.1 Da, smoothing method: linear weighted moving average, smoothing level = 3, minimum peak width = 5; (*iii*) alignment: retention time tolerance = 0.05 min, MS1 tolerance = 0.015 Da. We normalized the data using the total ion chromatogram function of MS-DIAL. We exported the alignment matrix as *.csv* file. The numbers in this matrix represent features defined by an average retention time and an average *m*/*z* value. We used the matrix for computing multivariate statistical analyses with the software *R* (v x64 3.5.1, *R* Foundation for Statistical Computing). We worked with the package *muma* for metabolomics (http://www.eurekaselect.com/107837) to calculate principal component analysis (PCA). We applied the function *pareto* for scaling the data.

# THESIS SUMMARY

## THESIS SUMMARY

The number of empirical studies on plant-mediated interactions between aboveground-belowground (AG-BG) herbivores is rapidly increasing. Despite this increased interest in AG-BG interactions, studies involving plant-parasitic nematodes have so far been under-represented. I aimed to investigate how the life cycle stages of root-knot nematodes (RKN) affect AG insect herbivores with differing feeding styles (chewing and piercing-sucking) via systemic induced plant responses. I hypothesized that the interaction between the RKN (*Meloidogyne incognita*) and two AG insect herbivores, the generalist caterpillar *Spodoptera exigua* and the specialist aphid *Macrosiphum euphorbiae*, as well as the underlying induced responses, depend on the nematodes' life cycle stages. To test this hypothesis, I set-up a series of experiments using tomato (*Solanum lycopersicum* cv. 'Moneymaker') as the model plant. I assessed the performance of the caterpillar and aphid when feeding on plants infected with the RKN at the invasion, galling, and reproduction stages. Using molecular and chemical analyses, I measured changes in phytohormones and metabolic profiles in roots and leaves from plants exposed to combinations of the RKN and one AG herbivore, at the three stages of the nematodes' life cycle stages (invasion, galling and reproduction).

The results obtained demonstrated that root infection by the RKN increased the performance of the caterpillar only when the nematode was at the galling stage. However, the RKN did not influence the performance of the aphid. These results indicate that that the effect of RKN on AG herbivorous insects depends on the feeding type of the AG insect herbivore and further supports the hypothesis that the nematodes' life cycle stage influence the outcome of AG-BG plant-mediated interactions. Next, I aimed to identify the mechanisms that might underlie these effects. Because phytohormones are induced with considerable specificity to the attacking herbivore, I analyzed the changes in jasmonates (JAs), salicylic acid (SA), abscisic acid (ABA), and the auxin (indole-3-acetic acid IAA) levels, and expression of defense signaling marker genes. Also, I measured the changes in the concentration of the steroidal glycoalkaloid (SGA): *α*-tomatine, which is the main allelochemical against herbivores produced in tomato plants. Also, I investigated changes in the expression of two steroidal glycoalkaloid metabolism (GAME) marker genes.

I found that RKN root infection increased the levels of JA, SA, and ABA locally in roots, and only SA systemically in leaves. The marker genes were not affected. The SGA levels and expression of GAME genes increased in roots but not in leaves. These inductions were most significant at the galling and reproduction stages, demonstrating that indeed the RKN modulates local and systemic responses depending on the infection cycle. The induction of phytohormones, SGA, and their related marker genes by the caterpillar and aphid was influenced by the host plant age as well as ontogeny. Locally in leaves, caterpillars triggered JAs, SA and ABA signaling and related marker genes more strongly in young (vegetative) plants compared to old (flowering) plants. The aphid decreased the levels of SGA and the expression of GAME

genes more in vegetative plants than in flowering plants. Systemically in the roots, caterpillar feeding downregulated the expression of GAME genes only in flowering plants. The aphid decreased the levels of JA, ABA, and IAA in the flowering plants compared to vegetative plants. The results on the effect of RKN on AG insect herbivore-induced responses showed that RKN infection broadly enhanced the JA-dependent induced response triggered by the caterpillar in leaves throughout the entire nematodes' infection cycle. These results suggest the principle of priming, as the RKN alone did not induce the JA pathway systemically in leaves. Aphid feeding on RKN-infected plants did not affect the phytohormonal pathways or related marker genes in leaves. However, SA levels, but not SA marker gene expression, were systemically induced in leaves by the RKN alone, and this did not change when aphids were feeding. These results indicate that the performance of the caterpillar and aphid did not decrease despite the increase in the levels of JAs and SA. Collectively, this suggests that other plant physiological processes might be involved in these interactions. Besides the phytohormones, RKN infection modified the SGA levels induced by the caterpillar and aphid in leaves. The results obtained revealed that the concentration of $\alpha$-tomatine increased during the early stages of RKN infection, whereas there was no effect of herbivore-induced SGA levels at later stages. The expression of GAME genes in leaves of RKN infected plants did not change upon herbivory by the insects. In the case of the caterpillar, I also found that a polyamine conjugated to a phenylpropanoid increased in plants when RKN and caterpillar co-occurred, particularly at the nematodes' galling stage. Whereas conjugated polyamines can be anti-herbivore molecules, this particular conjugate appeared not to affect the caterpillar's performance negatively. Further studies are required to elucidate the role of these metabolites in AG-BG interaction.

I also analyzed for the reciprocal effects of caterpillar and aphid leaf feeding on root induced responses to RKN throughout the entire life cycle. The results demonstrated that the impact of AG insect herbivory on RKN induced root responses depend on the type of insect herbivore, and the nematodes' root infection cycle stage. Caterpillar feeding on RKN- infected plants had differential effects on the JA pathway. The JA levels in roots decreased, but the expression levels of *Leucine aminopeptidase A (LapA)* gene were upregulated when caterpillars fed on plants in which the RKN was at the reproduction stage. Aphid feeding did not affect the root levels of phytohormones nor the expression of related defense marker genes triggered by RKN. Neither of the AG insect herbivores affected the levels of $\alpha$-tomatine nor the expression of the GAME genes triggered by the RKN locally in roots. These results show that RKN root infection induced the expression of GAME genes and SGAs production independent of the presence of AG insect herbivores.

In conclusion, the findings presented in this thesis provide evidence that the effect of RKN root infection on AG insect herbivores and AG-induced defense responses, as well as the effect of AG insect herbivores on root induced defense responses to RKN, are modulated by the nematodes' infection cycle.

The findings I presented here generate a better understanding of the molecular and chemical mechanisms that underlie frequent parasitic root nematode-plant-AG insect herbivore interactions in natural and agro-ecosystems.

# ZUSAMMENFASSUNG DER THESIS

**ZUSAMMENFASSUNG DER THESIS**

Die Zahl der empirischen Studien zu durch Pflanzen vermittelten Interaktionen zwischen oberirdischen („aboveground“; AG) und unterirdischen („belowground“; BG) Pflanzenfressern nimmt rasch zu. Trotz dieses gestiegenen Interesses an AG-BG-Interaktionen sind Studien mit pflanzenparasitären Nematoden bisher unterrepräsentiert. Ziel meiner Arbeit war es zu untersuchen, wie sich die Stadien im Lebenszyklus von Wurzelknotennematoden („root-knot nematode“; RKN) über systemisch induzierte Pflanzenreaktionen auf AG pflanzenfressende mit unterschiedlichen Ernährungsweisen (kauend und stechend-saugend) auswirken. Meine Hypothese lautete, dass die Interaktion zwischen dem RKN (*Meloidogyne incognita*) und zwei AG pflanzenfressenden Insekten, der generalistischen Raupe *Spodoptera exigua* und der spezialisierten Blattlaus *Macrosiphum euphorbiae*, sowie die zugrunde liegenden induzierten Reaktionen von den Lebenszyklusstadien der Nematoden abhängen. Um diese Hypothese zu überprüfen, habe ich mehrere Experimente mit der Modellpflanze Tomate (*Solanum lycopersicum* cv. 'Moneymaker') durchgeführt. Ich untersuchte die Entwicklung der Raupe und der Blattlaus beim Fraß von mit RKN infizierten Pflanzen im Invasions-, Gallenbildungs- und Reproduktionsstadium. Mit Hilfe molekularer und chemischer Analysen habe ich Veränderungen der Phytohormone und Stoffwechselprofile während dieser drei Stadien des Lebenszyklus von Nematoden in Wurzeln und Blättern von Pflanzen, die Kombinationen von RKN und einem AG-Pflanzenfresser ausgesetzt waren, gemessen.

Die erzielten Ergebnisse zeigten, dass eine Infektion der Wurzel durch RKN die Anfälligkeit der Wirtspflanze gegenüber der Raupe nur dann erhöhte, wenn sich die Nematoden im Gallenbildungsstadium befanden. Die RKN hatten jedoch keinen Einfluss auf die Entwicklung der Blattlaus. Diese Ergebnisse stützten die Hypothese, dass die Wirkung von RKN auf AG-pflanzenfressende Insekten von der Ernährungsweise des Insektes abhängig ist und weiterhin, dass die Lebenszyklusstadien der Nematoden die Wirkung von durch Pflanzen vermittelten AG-BG Interaktionen beeinflussen. Anschließend versuchte ich die Mechanismen zu identifizieren, die diesen Effekten zugrunde liegen könnten. Da Phytohormone mit einer erheblichen Spezifität für den angreifenden Pflanzenfresser induziert werden können, analysierte ich Veränderungen im Jasmonat- (JA), Salicylsäure- (SA), Abscisinsäure- (ABA) und Auxinspiegel (Indol-3-essigsäure IAA) sowie die Expression von Markergenen der pflanzlichen Abwehr-Signalkaskade. Außerdem habe ich die Veränderungen in der Konzentration des steroidalen Glykoalkaloids (SGA), α-Tomatine, gemessen. α-Tomatine ist die wichtigste Allelochemikalie gegen Pflanzenfresser, die in Tomatenpflanzen produziert wird. Weiterhin untersuchte ich Veränderungen in der Expression von zwei Markergenen des Steroidalglycoalkaloid-Stoffwechsels („glycoalkaloid metabolism“; GAME).

Ich fand heraus, dass eine RKN-Wurzelinfektion die Konzentrationen von JA, SA und ABA lokal in den Wurzeln erhöhte und nur SA systemisch in den Blättern induziert wurde. Die Markergene waren nicht betroffen. Die SGA-Konzentrationen und die Expression der GAME-Gene erhöhten sich in den

Wurzeln, aber nicht in den Blättern. Diese Induktionen waren vor allem im Gallenbildungs- und Reproduktionsstadium von Bedeutung, was zeigt, dass RKN in der Tat lokale und systemische Reaktionen in Abhängigkeit vom Infektionszyklus modulieren. Die Induktion von Phytohormonen, SGA und ihren dazugehörigen Markergenen durch Raupe und Blattlaus wurde sowohl durch das Alter der Wirtspflanze als auch durch die Ontogenie beeinflusst. Raupenfraß erhöhte lokal in den Blättern die JA-, SA- und ABA-Signalkaskade und die Expression verwandter Markergene bei jungen (vegetativen) Pflanzen stärker aus als bei alten (blühenden) Pflanzen. Die Blattlaus verringerte die SGA Konzentration und die Expression von GAME-Genen in vegetativen Pflanzen stärker als in blühenden Pflanzen. Raupenfrass regulierte systematisch in den Wurzeln die Expression der GAME-Gene nur bei blühenden Pflanzen herunter. Die Blattlaus verringerte die Konzentrationen von JA, ABA und IAA in den blühenden Pflanzen im Vergleich zu vegetativen Pflanzen. Die Resultate zum Einfluss von RKN auf die durch AG pflanzenfressenden Insekten induzierte Pflanzenreaktion zeigten, dass die RKN-Infektion die JA-abhängige Raupen-induzierte Reaktion, die durch die Raupe in den Blättern ausgelöst wurde, während des gesamten Infektionszyklus der Nematoden weitgehend verstärkte. Diese Ergebnisse legen das Prinzip des Priming nahe, da RKN allein den JA-Signalweg in den Blättern nicht systemisch induzierte. Blattläuse, die sich von RKN-infizierten Pflanzen ernährten, hatten keinen Einfluss auf die phytohormonalen Pfade oder verwandten Markergene in den Blättern. Allerdings wurde in Blättern die SA-Konzentration, nicht aber die Expression von SA-Markergenen, systemisch allein durch RKN induziert. Diese änderte sich auch nicht, wenn die Pflanzen von Blattläusen befallen waren. Diese Ergebnisse deuten darauf hin, dass die Leistung der Raupe und der Blattläuse trotz des Anstiegs der JA- und SA-Konzentrationen nicht abnahm. Zusammengenommen weist dies darauf hin, dass andere pflanzenphysiologische Prozesse an diesen Interaktionen beteiligt sein könnten. Neben den Phytohormonen veränderte die RKN-Infektion die SGA-Werte, die durch die Raupe und die Blattlaus in den Blättern induziert wurden. Die erzielten Ergebnisse zeigten, dass die Konzentration von α-Tomatine während der frühen Stadien der RKN-Infektion zunahm, während es in späteren Stadien keinen Effekt der durch Pflanzenfresser induzierten SGA-Konzentrationen gab. Die Expression der GAME-Gene in Blättern von RKN-infizierten Pflanzen änderte sich bei Fraß durch die Insekten nicht. Im Fall der Raupe fand ich zusätzlich auch heraus, dass ein mit einem Phenylpropanoid konjugiertes Polyamin in Pflanzen zunahm, wenn RKN und Raupe gleichzeitig auftraten, insbesondere im Gallenbildungsstadium der Nematoden. Während konjugierte Polyamine Anti-Pflanzenfresser-Moleküle sein können, schien dieses spezielle Konjugat die Performance der Raupe nicht negativ zu beeinflussen. Weitere Studien sind erforderlich, um die Rolle dieser Metaboliten bei der AG-BG-Interaktion zu klären.

Des Weiteren analysierte ich die gegenseitigen Auswirkungen des Fraßes von Raupe und Blattlaus auf die durch RKN induzierten Reaktionen in Wurzeln während des gesamten Lebenszyklus. Die Ergebnisse zeigten, dass der Einfluss von AG-pflanzenfressenden Insekten auf RKN-induzierte

Wurzelreaktionen von der Art des pflanzenfressenden Insektes und dem Stadium des Wurzelinfektionszyklus der Nematoden abhängt. Raupen, die auf von RKN-infizierten Pflanzen fraßen, hatten unterschiedliche Auswirkungen auf die JA-Signalkaskade. Die JA-Konzentration in den Wurzeln sank, aber die Expressionswerte des Leucin-Aminopeptidase A (LapA)-Gens wurden hochreguliert, wenn die Raupen sich von Pflanzen ernährten, in denen sich das RKN im Reproduktionsstadium befand. Der Blattlausbefall beeinflusste weder die Konzentrationen der Phytohormone in der Wurzel noch die Expression verwandter Abwehr-Markergene, die durch die RKN ausgelöst wurden. Weder die AG-fressenden Insekten beeinflussten die Konzentration von α-Tomatine noch die Expression der durch die RKN ausgelösten GAME-Gene lokal in den Wurzeln. Diese Ergebnisse zeigen, dass eine RKN-Wurzelinfektion die Expression von GAME-Genen und die Produktion von SGAs unabhängig von der Anwesenheit von AG-fressenden Insekten induzierte.

Zusammenfassend zeigen die in dieser Arbeit vorgestellten Ergebnisse, dass die Wirkung der RKN-Wurzelinfektion auf AG-pflanzenfressende Insekten und AG-induzierte Abwehrreaktionen sowie die Wirkung von AG-pflanzenfressenden Insekten auf Wurzel-induzierte Abwehrreaktionen gegen RKN durch den Infektionszyklus der Nematoden moduliert werden. Die von mir hier vorgestellten Ergebnisse führen zu einem besseren Verständnis der molekularen und chemischen Mechanismen, die den verbreiteten Interaktionen zwischen parasitären Wurzelnematoden, Pflanzen und oberirdisch pflanzenfressenden Insekten in natürlichen und Agrarökosystemen zugrunde liegen

# DECLARATION OF INDEPENDENT ASSIGNMENT

## DECLARATION OF INDEPENDENT ASSIGNMENT

Per the requirements and regulations laid down for doctoral studies in the Faculty of Biological Sciences of the Friedrich Schiller University in Jena Germany, I confirm that: I am familiar with the valid doctoral examination regulations. I have composed and written the doctoral thesis submitted myself. I did not use any section(s) of text or image(s) from third parties nor their previous thesis without proper citation of the source. Also, I confirm that I have cited the tools, personal communication, and sources I have been using. I have provided names of the persons who assisted in selecting and analyzing materials and supported them in writing. I did not receive any assistance from a specialized consultant, and no third party who received either direct or indirect financial benefits for work connected to the doctoral thesis submitted. I have not submitted the doctoral thesis project as the final thesis for a state examination or other examination. I have not submitted the doctoral thesis or a substantially similar or another scientific paper to any other institution of higher education or any other faculty as a doctoral thesis.

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## EIGENSTÄNDIGKEITSERKLÄRUNG

Gemäß den Anforderungen und Vorschriften für das Promotionsstudium an der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bestätige ich dies: Ich bin mit der geltenden Promotionsordnung vertraut. Ich habe die eingereichte Doktorarbeit selbst verfasst und geschrieben. Ich habe keine Text- oder Bildabschnitte, Bilder von Dritten oder deren früheren Arbeiten ohne ordnungsgemäße Quellenangabe verwendet. Ich bestätige auch, dass ich die von mir verwendeten Hilfsmittel, persönlichen Mitteilungen und Quellen zitiert habe. Ich habe die Namen der Personen angegeben, die bei der Auswahl und Analyse der Materialien geholfen und sie schriftlich unterstützt haben. Ich habe keine Hilfe von einem spezialisierten Berater erhalten und keine dritte Person, die entweder direkt oder indirekt finanzielle Zuwendungen für Arbeiten im Zusammenhang mit der eingereichten Doktorarbeit erhalten hat. Ich habe das eingereichte Dissertationsprojekt nicht als Abschlussarbeit für ein Staatsexamen oder eine andere Prüfung eingereicht. Ich habe die Doktorarbeit oder eine im wesentlichen ähnliche oder eine andere wissenschaftliche Arbeit nicht als Dissertation bei einer anderen Hochschule oder einer anderen Fakultät eingereicht.

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# ACKNOWLEDGEMENTS

## ACKNOWLEDGEMENTS

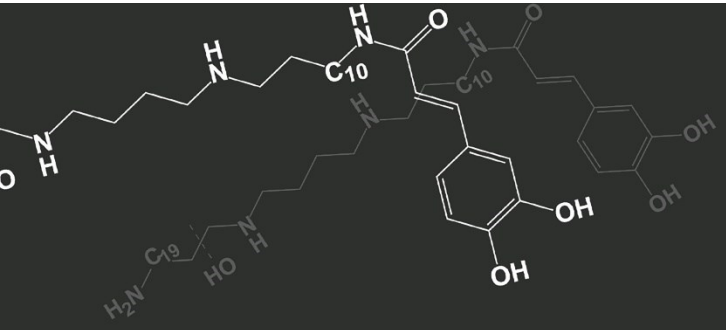
First and foremost, I thank God, for all-round care of my well-being throughout my Ph.D. challenge. Second, I would like to acknowledge the following persons who supported me when I was in the process of searching for a Ph.D. position/program. I want to thank Dr. Beena M. Ravindran, Dr. Calvin Onyango, and Dr. Jagger Harvey. You all advised and encouraged me, and wrote many recommendation letters to support my pursuit of a Ph.D. position. Third, I would like to thank my supervisors for doctoral studies: Prof. Dr. Nicole M. van Dam, and Dr. Ainhoa Martínez-Medina. I would imagine that supervision is like a puzzle without all pieces! Yet you have to fix it. The doors to your offices were always open whenever I had questions about my research, writing, or at times personal matters, you tirelessly listened, advised, and guided me in the right direction to achieve my goal within the set time. Without your support and dedicated involvement in every step throughout my Ph.D. journey, my thesis would not have been accomplished. Once again, I say thank you and God's blessings. I want to extend my gratitude to my Ph.D. advisory committee (PAC), including Prof. Dr. Nicole M. van Dam, Dr. Ainhoa Martínez-Medina, Dr. Meredith Schuman, and Prof. Dr. Nico Eisenhauer. Every time I had my PAC meeting it was a chance to step up my 'A-GAME' into building my career in plant sciences. Thank you for turning up for the meetings, insights, and tips on how to improve myself career wise and even personally, and for the encouragement to keep the focus on the ultimate goal, Ph.D.

Next, I would like to thank several members of the Molecular Interaction Ecology (MIE) group whom I worked directly with or participated in parts of my doctoral research work. I thank Dr. Katharina Grosser, Dr. Fredd Vergara, and Anne Mädicke for the training/teachings and support on chemical and molecular biology analyses that I needed for my work. The gained knowledge will go beyond my thesis into future endeavors for a prosperous career in plant sciences. In addition, I would like to thank Dr. Katharina Grosser for always accepting to remotely keep an eye on me when I had to work in the laboratory over weekends. That was kind of you, and God bless you. Besides the MIE laboratory, I did many experiments in the glasshouse at the botanical gardens of the University Leipzig. I want to thank Daniel Uhlig, and Alvin Quentin Barth both who supported me in setting up things for my experiments in the glasshouse. I also thank Dr. Simone Cesarz and Alfred Lochner both from the Experimental Interaction Ecology group at iDiv. I borrowed some items that I needed for my work from your laboratory and consulted at times. I say thank you.

I extend my sincere appreciation to Jennifer Gabriel for the graphics and artwork on some figures in my thesis. Also, I thank you for the translations of some thesis texts from English to German. On the same note, I thank Dr. Alexander Weinhold, it was easy to talk to you, and that made me feels good. Thank you also to the translations of English to German. You helped me on several occasions, some of which were not even work-related. I would like to thank Susann Goerth. Your position in the group is crucial from the beginning of one’s contract till the end. I want to thank you for your help in paperwork, reminder checks on my budget and many other things. The MIE laboratory is not only composed of the few names mentioned above, but there are also many others: some who left and others

still around, allow me to thank you all. I had an opportunity to supervise two students Esraa M. Ahmad for a Master of science thesis and Melody Fu for Bachelor internship. By working with you I grew my teaching and leadership skills. I thank you for taking part in my research project.

Getting through my Ph.D. journey required more than just the academic environment, I have many, many other people to thank for being there for me, listening, tolerating me for the past four-plus years. I just want to say thank you to you all. Family is a vital part of everyone's life; I was blessed to have one, your love and prayers kept me going till the end. I say thank you and God bless you all. Lastly, I want to thank Deutscher Akademischer Austauschdienst (DAAD), German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, yDiv graduate school, and Friedrich Schiller Universität Jena (FSU-Jena). All these institutions and persons working in various positions supported my Ph.D. journey in and at several levels. I also want to thank you all.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